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Role of hepatitis B virus in non-B, non-C chronic liver disease: in vitro proliferation and interferon-gamma production of peripheral blood mononuclear cells in response to hepatitis B core antigen and its relation to hepatitis activity

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Title

Role of hepatitis B virus in non-B, non-C chronic liver disease: *in vitro* proliferation and interferon-gamma production of peripheral blood mononuclear cells in response to hepatitis B core antigen and its relation to hepatitis activity.

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Abstract

Objective: Although hepatitis B virus (HBV) DNA has been detected in the sera of patients with chronic liver disease with neither hepatitis B surface antigen nor anti-hepatitis C virus antibody [non-B, non-C (NBNC) CLD], whether HBV has some pathogenic role in NBNC CLD has not been made clear. ***Methods:*** To investigate the significance of HBV DNA in NBNC CLD, we performed *in vitro* stimulation assays of peripheral blood mononuclear cells (PBMCs) in response to hepatitis B core antigen (HBcAg) in 17 NBNC CLD patients. ***Results:*** HBV DNA with an eight-nucleotide deletion in the core promoter region was detected in 13 (76%) of the 17 patients by nested polymerase chain reaction. Interferon-gamma (IFN-γ) production and proliferation of PBMCs of HBV DNA-positive patients showed a significant increase in response to HBcAg. The histological activity of hepatitis was also found significantly associated with the magnitude of IFN-γ production and proliferation of PBMCs in response to HBcAg. Although 5 (38%) of the 13 HBV DNA-positive NBNC CLD patients had anti-HBs and/or anti-HBc, there was no difference in response of PBMCs to HBcAg between the HBV DNA-positive and negative groups. ***Conclusion:*** Our observation suggests that HBV may have a pathogenic role in HBV DNA-positive NBNC CLD, even in those patients without any serological markers of HBV.

Key words: Non-B non-C chronic hepatitis, HBV DNA, PBMCs, IFN-γ, HBcAg.

Introduction

Hepatitis B virus (HBV) DNA may be deteted at very low levels in the sera of patients with acute hepatitis B, even decades after complete resolution (1-3). Since these patients have antibodies to HBV antigens and HBV DNA in serum together with a normalized serum alanine aminotransferase (ALT) level, the existence of such a low level of HBV DNA itself has been considered to have no significance, other than a marker of remote infection. However, the significance of persistent low level HBV DNA in the serum of patients with chronic liver disease without hepatitits B surface antigen (HBsAg) in serum has not been made clear. Many studies have shown that a very low level of HBV DNA can be detected by nested polymerase chain reaction (PCR) in the serum or liver tissue of patients with chronic liver disease (CLD) who exhibit neither HBsAg nor anti-hepatitis C virus antibody (anti-HCV) in serum [non-B, non-C (NBNC) CLD] (4-9). Interestingly, about two thirds of these patients do not exhibit any of the serological markers of HBV infection despite the persistence of HBV DNA in serum (4,5,8). Therefore, further studies is needed to determine whether the persistence of a low level of HBV has any pathogenic role in NBNC CLD patients.

HBV has no direct cytopathic effect on infected hepatocytes, but the host cellular immune response to HBV is responsible for the hepatocellular damage (10). In chronic hepatitis B (CH-B), T cells proliferate and produce interferon gamma (IFN-γ) *in vitro* in response to hepatitis

B core antigen (HBcAg), which has been shown as the major target antigen recognized by cytotoxic T cells (11-17). Moreover, the magnitude of the immune response to HBcAg is usually correlated to the histological activity of hepatitis in CH-B (18-20). On the contrary, T cells from hepatitis B e antigen (HBeAg)-positive healthy carriers without inflammation in the liver do not respond to HBcAg (17,19). In this context, the *in vitro* stimulation assay of T cells using HBcAg appears to be a useful method to reveal whether patients of NBNC CLD can recognize HBV immunologically. In addition, if the degree of immune response to HBcAg has a clear association with the histological activity of hepatitis in these patients, HBV might have some implications in the pathogenesis of NBNC CLD. Accordingly, we investigated the *in vitro* proliferation and IFN-γ production of peripheral blood mononuclear cells (PBMCs) in response to HBcAg to understand the significance of low level HBV in NBNC CLD.

Materials and Methods

Patients

Seventeen patients with well-characterized NBNC CLD were the subjects of this study. These patients had been followed at the Shimane Medical University Hospital and other related hospitals for 2 to 15 years with a mean observation period of 5.4 years. These patients had no history of either acute hepatitis B or prior diagnosis as a chronic HBV carrier. They had no history of blood

transfusion or previous HBV vaccination, nor was there any family history of HBV-associated liver disease was seen in any of these patients. Furthermore, there was no evidence of habitual alcohol abuse nor metabolic diseases including diabetes mellitus, Wilson's disease, or hemochromatosis. All patients had mildly elevated serum ALT levels (40 to 146 IU/L; normal range ≤35) during the follow-up period. HBsAg and the second-generation anti-HCV were not detected in these patients, and serum HBV DNA polymerase activity and HBV DNA, as confirmed by the usual spot hybridization, were also negative during the follow-up period, even though these markers were tested for at least three times every year. In addition, these patients had no significant titer (less than 40x) of autoantibodies including anti-nuclear antibody, anti-smooth muscle antibody, or anti-mitochondrial antibody.

Control patients

As disease controls, 16 CH-C and 12 CH-B (including 8 HBeAg-positive and 4 anti-HBe-positive) patients were included. As negative controls, 33 age/sex-matched healthy volunteers were also included. The characteristics of these patients are shown in Table 1.

Identification of HBV DNA in serum

We and Uchida et al. have previously revealed independently that HBV, which has an eight-nucleotide (nt) deletion in the core promoter region, is a virological agent characteristic of HBV-positive NBNC CLD (7,9). Thus, to

investigate whether these patients were infected with HBV or not, HBV DNA fragments, including the core promoter region, were amplified by two-stage nested PCR according to our previous method (9). Briefly, 300μl of serum was digested with proteinase K and DNA was extracted from it by the phenol/chloroform method. The primers for the first-stage PCR were; p202 5'-CTGCCGTTCCGGCCGACCAC-3' (sense) and H2 5'-AGAAAAAACGGAAGACTGAA-3' (anti-sense). Those for the second-stage PCR were; H1 5'-CATAAGAGGACTCTTGGACT-3'(sense) and 201 5'-ATTAGGCAGAGGTGAAAAAG-3' (anti-sense). The amplified DNA band was confirmed with the aid of an ultraviolet lamp after gel electrophoresis containing ethidium bromide. PCR was performed twice in each sample on different days. The PCR result was defined as positive or negative if both PCRs gave positive results or negative results, respectively. If only one PCR was positive, an additional PCR was performed, and the result of this additional PCR was considered to be final. The DNA sequences of the amplified fragments were analyzed by direct sequencing as previously reported (9).

Serological study for viral markers in serum

HBsAg (Radio immunoassay, ABBOTT LABORATORIES, Chicago, IL), anti-HBs (Radioimmunoassay, ABBOTT), anti-HBc (Radioimmunoassay, ABBOTT), hepatitis B e antigen (HBeAg; Enzyme immunoassay, ABOTT), anti-HBe (Enzymeimmunoassay, ABOTT) and second-generation anti-HCV (Dinabott, Tokyo) were tested using patients' sera. Determination of the seropositivity or seronegativity of these markers was

according to the instruction manuals of the kits. The usual spot hybridization assay for HBV DNA (21), reverse transcription-PCR for both HCV RNA (22), and hepatitis G virus RNA (23) were performed for viral nucleic acid detection.

DNA sequencing

Amplified HBV DNA was purified from the agarose gel using a Gene clean II Kit (BIO 101,Inc. Vista CA), and directly sequenced using ^{35}S according to our previous method (9).

Histological examination

A liver biopsy was performed in 15 of the 17 patients with NBNC CLD. A biopsy could not be obtained from two patients because of their old age. In the control groups, a liver biopsy was obtained from 12 CH-B patients and 16 CH-C patients during the present study. The liver specimens were fixed in 10% buffered folmarin and embedded in paraffin followed by hematoxylin and eosin staining. The histological activity of necroinflammation was expressed using Knodell's histological activity index score (HAI score) category I to III (24).

Immunohistochemical study

HBsAg and HBcAg were investigated by immunostaining in the 15 NBNC patients and 12 CH-B patients. The liver sections were deparafinized and reacted with the first antibody (anti-HBs mouse monoclonal antibody and anti-HBc rabbit monoclonal antibody (DAKO Japan Co.Ltd, Tokyo) at 4^{o} C

overnight. The sections were then reacted with a second antibody (goat anti-mouse or anti-rabbit IgG conjugated horseradish peroxydase, DAKO) at 37^{o} C for 1 hour. The reaction products were visualized by treatment with 3,3'-diaminobenzidine tetrahydrochloride. As negative control, immunostaining using normal mouse and rabbit serum as the first antibody was also performed.

Preparation of PBMCs

We used unseparated PBMCs to investigate the host immune response for *in vitro* stimulation. Unseparated PBMCs were isolated from all of the subjects using heparinized venous blood by Ficol-Hypaque density gradient centrifugation. The PBMCs at the interphase were washed three times in RPMI-1640 culture medium (Gibco, Grand Island, NY) and suspended at 1 x 10^{6} cells/ml in RPMI-164 medium with 10% heat-inactivated fetal bovine serum and 20μg/ml of glutamin.

Determination of subpopulation of PBMCs

The subpopulation of PBMCs were determined by indirect immunofluorescence flow cytometry using the following monoclonal antibodies from Nihon Beckton Dikkenson, Co.Ltd (Tokyo, Japan); Leu-3a for $CD4^{+}$ T cells, Leu-2a for $CD8^{+}$ T cells, Leu-16 for $CD20^{+}$ B cells, and Leu-19 for $CD56^{+}$ NK cells.

Additives

Recombinant HBcAg, obtained from a bacterial extract of *Escherichia coli* (*E.coli*) JM83, was kindly provided by the Institute of Microbiology of Osaka University (Kanonji, Japan). Concanavalin A (ConA) was purchased from Gibco.

IFN-γ production by PBMCs

In order to investigate the response of PBMCs to HBcAg, PBMCs containing $2x10^6$ cells were cultured in duplicate for 5 days with 1 μg/ml of HBcAg. This condition was determined by a preliminary study in which the concentrations of HBcAg and culture periods were varied. PBMCs were cultured in 96-well culture plates at 37° C in a 5% CO_2/ 95% air incubator. The supernatants from the culture medium after centrifugation were collected and stored at -20° C until measurement of IFN-γ. As positive control, PBMCs were cultured with 50μg/ml of Con A. As negative control, *E.coli* JM83 extract (3μg/ml) was used for a stimulation assay. IFN-γ was measured by using a commercial enzyme-linked immunosorbent assay (ELISA) kit (PREDICTA IFN-γ ELISA Kit, Genzyme, Co.Ltd, Cambridge, MA) using 100 μl of the culture supernatant. The mean value of duplicated samples was defined as the amount of IFN-γ production for each patient. To compare the IFN-γ production between patients, the stimulation index of INF-γ production was used for both ConA and HBcAg (SI; IFN-γ after stimulation /IFN-γ in medium without stimulation) was used.

Proliferation assay

The proliferation of PBMCs was measured using a commercial kit (Cell proliferation ELISA system, version 2. Amersham International plc). Briefly, PBMCs of $1x10^5$ cells/well were cultured in flat-bottomed 96-well microtiter plates, in the culture medium for 4 days at 37° C in a humidified 5% CO_2 / 95% air incubator in the presence of 1µg/ml HBcAg. The culture was further incubated for 24 hours with 10 µM bromodeoxyuridine (BrdU). After incubation, the cells were collected and reacted with peroxidase-labeled anti-BrdU antibody. The antibody was visualized, followed by the addition of tetrametylbenzidine and the absorbance at 450 nm was measured using a spectrophotometer. The proliferation index to both ConA and HBcAg (PI; proliferation after stimulation /proliferation without stimulation) was used for the comparison of proliferation between disease groups. As positive and negative controls, PBMCs were cultured with 50µg/ml of Con A and 3µg/ml *E.coli* JM83 extract.

Statistical analysis

Statistical analyses were assessed by a χ^2-test by Fishers exact test, Student's unpaired t-test, and the Wilcoxons sum-rank test. The correlation between SI and PI to HBcAg was assessed by Peason's correlation coefficient. The level of statistical significance was set at $p<0.05$ for all analyses.

Results

Prevalence of HBV DNA in serum

The HBV DNA fragments were successfully amplified in 13 (76.5%) of the 17 NBNC patients at the second-stage PCR, while none of the patients showed HBV DNA at the first-stage PCR. Thus, we defined these 13 patients as HBV-positive NBNC CLD and the other 4 patients as HBV-negative NBNC CLD in the present study.

DNA sequences of amplified HBV DNA

The DNA sequence was investigated in 13 HBV-positive NBNC CLD patients and 5 patients with HBeAg-positive CH-B. As reported previously (7,9), a characteristic 8-nucleotide (nt) deletion mutation in the core promoter was observed in all of the DNA amplified from the 13 patients with HBV-positive NBNC CLD (Fig.1). However, this mutation was not found in the HBeAg-positive patients.

Serological results

Table 2 shows the serological data of the 17 patients with NBNC CLD. HBsAg and HBeAg were negative in all of the patients. In the 13 HBV-positive patients, 5 (38.5%) patients had antibodies to HBV. Two patients (patient #1 and #2) had both anti-HBc and anti-HBs. One patient (patient #3) had both anti-HBc and anti-HBe. One patient (patient #4) had only anti-HBc and the other patient (patient #5) had only anti-HBs. In the 4 HBV-negative patients, one patient (patient #14) had only anti-HBc and one patient (patient #15) had anti-HBc, anti-HBs, and anti-

HBe. Anti-HCV was negative in all of the patients. Neither HCV RNA nor HGV RNA was detected in any of the 17 patients. HBV DNA was not detected by a spot hybridization test (Data not shown).

Histological diagnoses of the patients with NBNC chronic hepatitis

The histological diagnosis of the 15 NBNC CLD patients was chronic hepatitis. However, hepatitis activity expressed as category I to III of the HAI score was weak in HBV-positive NBNC CLD (3.3±1.7 ;mean±SE) as compared to CH-B (5.3±0.7, $p<0.05$) and in CH-C (4.2±0.7). Although the HAI score was higher in HBV-positive CLD patients compared with HBV-negative patients (2.3±0.9), it was not statistically significant.

HBsAg and HBcAg in the liver

Out of the 13 HBV-positive NBNC CLD patients, HBsAg could be detected in 8 (61.5%) and HBcAg in 4 (30.7%) liver tissues. However, neither HBsAg nor HBcAg could be detected in the liver tissue of HBV-negative NBNC CLD patients. HBsAg was stained in the cytoplasma of the hepatocytes with a weak intensity and distributed either focally or scattered throughout the biopsy samples (Fig.2.a). HBcAg was seen only in a few nuclei of the hepatocytes (Fig.2.b). HBcAg-positive hepatocytes were far less frequent as compared with HBsAg-positive hepatocytes. In patients with CH-B, all 12 had HBsAg and HBcAg in the liver with variable intensity (data not shown). Positive signals

were not obtainied in staining using the normal mouse and rabbit serum as the 1st antibodies as negative controls.

Subpopulation of PBMCs

No significant difference in the subpopulation of PBMCs obtained was seen among the disease groups (data not shown).

IFN-γ production of PBMCs in response to HBcAg

Stimulation indexs (SIs) of IFN-γ production in different disease groups are shown in Table 3. In response to HBcAg, the SI was significantly increased in HBV-positive NBNC CLD as well as CH-B, while no significant increase in SI was seen in CH-C patients or healthy controls. There was no statistical difference in the SI between patients with CH-B and those with HBV-positive NBNC CLD. The SIs of CH-C patients and healthy controls were significantly lower compared with those of both CH-B (both $p<0.0001$) and HBV-positive NBNC CLD (vs CH-C $p<0.0001$, vs healthy volunteers $p<0.001$). The SI of patients with HBV-negative NBNC CLD was also lower compared with those with HBV-positive NBNC CLD. In response to ConA, SIs of IFN-γ production were lower in both CH-B ($p<0.001$) and HBV-positive NBNC CLD patients ($p<0.05$) as compared to the control group. No significant response of PBMCs was seen in response to the *E.coli* JM83 extract (data not shown).

Proliferation of PBMCs in response to HBcAg

The proliferation response of PBMCs to HBcAg was also similar to the IFN-γ response (Table 3). The PIs in response to HBcAg in CH-B patients and those in HBV-positive NBNC CLD were significantly higher than for those CH-C ($p<0.0001$) and healthy controls ($p<0.0001$). The PI was lower in patients with HBV-negative NBNC CLD compared with HBV-positive NBNC CLD. Proliferation index (PI) to Con A were not different among the disease groups. *E.coli* JM83 extract did not cause significant proliferation of PBMCs (data not shwon).

Relationship between proliferation and IFN-γ production of PBMCs to HBcAg

In both CH-B and HBV-positive NBNC CLD patients, the SIs of IFN-γ production were significantly correlated to the PIs of PBMCs assessed by Peason's correlation coefficient ($r=0.57$, $p<0.01$ and $r=0.76$, $p<0.001$, respectively).

Histological activity of hepatitis and immune response to HBcAg

The histological activity of hepatitis evaluated by the HAI score in HBV-positive NBNC CLD was significantly correlated with both SI and PI of PBMCs in response to HBcAg (Fig.3). However, there was no association between the immune response to HBcAg and hepatitis activity in HBV-negative NBNC CLD. In CH-B, there was also a significant correlation between the HAI score and immune response to HBcAg (Fig.3).

Clinical and immunological differences between patients with and without antibodies to HBV

In HBV-positive NBNC CLD, patient age was not significantly different between the two groups. Patients without antibodies also showed a clear immune response to HBcAg as those with antibodies did. Similarly, the HAI score was not different between the two groups (Table 4). Positivity of HBsAg and HBcAg in the liver was not different between these two groups.

Discussion

Although serum HBV DNA was identified in 13 (76.5%) of the 17 patients with NBNC-CLD in the present study, 8 (61.6%) of the 13 patients did not exhibit any serological markers of HBV, as previously reported (4,5,8). However, except for the serological results, both groups showed similar characteristics including identification of serum HBV DNA, a significant response of PBMCs to HBcAg, histologically-proven chronic hepatitis, and immunostaining of HBsAg and HBcAg in hepatocytes. Interestingly, all of these findings are also the characteristics of patients with CH-B. Accordingly, these results suggest that, irrespective of serological markers, HBV-positive chronic hepatitis patients with a significant response to HBcAg may have similar immunopathogical conditions in response to HBV. Although we used unseparated PBMCs to investigate the immune response to HBcAg, it has been shown that *in vitro* proliferation and IFN-γ production in response to HBcAg by

PBMCs represent that of T cells in CH-B (25,26). Although, PIs in the present study were lower relative to previous reports, this may be due to the difference in the method measuring the proliferation of PBMCs. In the present study, incorporated BrdU was detected by ELISA instead of [^{3}H]-thymidine incorporation. The method requires immunological reactions after cell lysis, including BrdU/anti-BrdU-antibody and anti-BrdU-antibody/peroxydase-conjugated second antibody. These multiple steps may result in a relatively lower sensitivity of this method as comapred to those using radioisotope.

Concernig the seronegativity of HBV markers in HBV-positive NBNC CLD patients, several possibilities may be considered. First, a low level of HBV markers may be below the detection level of conventional detection kits. Infection with silent mutant HBV may not exhibit any of the HBV markers from the beginning due to the extremely low capacity of replication. The 8-bp deletion mutant HBV has been associated with HBV infection in which the viral amount is extremely small (7,9,27). The importance of the X gene in HBV replication has been shown in many reports (28-35). Second, some other mutations in epitopes recognized by the antigens and antibodies used in commercial kits may result in the false negativity, since such mutations have been reported as the cause of discrepancy between serological and PCR results (36-38). Third, the antibodies to HBV may have already disappeared from the serum in two thirds of these patients in the long period of time

following HBV infection. Several combinations of these possibilities may also be considered.

Whether a small amount of HBV has a pathogenetic role in chronic inflammation in these NBNC CLD patients has not yet been clarified, despite the frequent identification of HBV DNA. It may not be excluded completely that the low level viremia of HBV and chronic hepatitis are mere coincidences. However, HBV DNA was not idnetified even in over double the number of age/sex-matched healthy controls included from the same area. In addition, the degree of immune response of PBMCs to HBcAg was significantly associated with the histological activity in these patients. Together with the existence of HBsAg and HBcAg in the liver in these patients, this suggests that HBV may be closely associated with inflammation in the liver. It has been reported that, even in patients with chronic hepatitis after seroconversion of HBsAg to anti-HBs, hepatitis activity was more severe in patients with serum HBV DNA as compared to those without HBV DNA in serum (39,40). This may also support a pathogenetic role of HBV in NBNC CLD, particularly in those patients who are found positive for anti-HBc and anti-HBs.

Although some discrepancy between the degree of immune response to HBcAg and the existence of HBcAg in the liver was observed, this may be due to the small size of the liver biopsy specimens. Since HBcAg does not usually exist diffusely throughout the lobules, even in patients with CH-B, it seems convincing that the negativity of HBcAg in the liver of our subjects does not exclude the possibility of a

positive finding on a repeated liver biopsy in patients who show a significant response to HBcAg. Uchida et al. has also reported that HBcAg was seen in only a few nuclei of hepatocytes in the liver of patients with silent mutant HBV infection (41). In patients with acute hepatitis B, both low levels of HBV DNA in serum and a strong immune response of T cells to HBcAg are maintained, even decades after the clinical resolution (1-3). However, all of these patients have anti-HBs and/or anti-HBc in their serum. Moreover, they demonstrate no necroinflammatory reactions in the liver, whereas our HBV-positive NBNC CLD patients had persistent necroinflammation in the liver with the presence of HBV antigens in the hepatocytes. Thus, the immune response to HBcAg in HBV-positive NBNC CLD may represent a different pathologial condition from that of post self-limited acute hepatitis B

Although our results suggest the possibility that HBV can be an immunopathological pathogen in NBNC CLD, further investigation, including isolation of HBV-specific cytotoxic T cells from the liver of these patients, is necessary to substantiate our findings.

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Table 1 Characteristics of the subjected patients and controls in this study.

#Group	†Number	Sex(M/F)	¶Age	§ALT(IU/L)
HBV-positive NBNC CLD	13	9/4	57.0±5.4	53.8±12.8
HBV-negative NBNC CLD	4	2/2	56.8±4.1	40.8±11.2
CH-B	12	10/2	35.4±2.8	264.8±85.0
CH-C	16	11/5	48.9±2.9	60.6±9.5
Control	33	20/13	54.0±3.0	25.8±2.3

#HBV-positive NBNC CLD, HBV DNA-positive non-B non-C chronic liver disease; HBV-negative NBNC CLD, HBV-negative non-B, non-C chronic liver disease; CH-B, chronic hepatitis B; CH-C, chronic hepatitis C; Control, healthy volunteers. †Number of cases. ¶Years old (mean±SE), §ALT: Alanine aminotransferase in serum ; mean±SE. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Table 2 Serological and biochemical test results in comparison with the response of PBMCs to HBcAg stimulation in HBV-positive and negative NBNC CLD.

No	Age/ Sex	#HBV DNA	HBsAg /Ab	HBeAg /Ab	Anti-HBc	ALT IU/L	§HBsAg /HBcAg	†SI	¶PI
1	38M	+	-/+	-/-	+	55	+/+	2.0	1.4
2	53F	+	-/+	-/-	+	72	+/-	10.3	1.6
3	80M	+	-/-	-/+	+	85	NT	10.5	1.8
4	53M	+	-/-	-/-	+	33	+/+	9.0	1.9
5	60M	+	-/+	-/-	-	38	-/-	2.3	1.3
6	60M	+	-/	-/-	-	85	+/-	5.2	1.4
7	75F	+	-/-	-/-	-	68	NT	11.5	2.3
8	29M	+	-/-	-/-	-	40	-/-	1.8	0.9
9	65M	+	-/-	-/-	-	59	+/-	7.2	1.4
10	50F	+	-/-	-/-	-	86	+/+	11.7	1.9
11	51F	+	-/-	-/-	-	61	+/-	8.0	1.5
12	55M	+	-/-	-/-	-	28	+/+	1.2	1.2
13	72M	+	-/-	-/-	-	28	-/-	12.5	1.7
14	54M	-	-/-	-/-	+	66	-/-	1.5	1.1
15	62M	-	-/+	-/+	+	17	-/-	1.3	1.3
16	44F	-	-/-	-/-	-	11	-/-	1.9	1.6
17	67M	-	-/-	-/-	-	43	-/-	1.2	0.8

#Nested PCR, §Immunostaining of HBsAg and HBcAg in the liver, +, positive; -, negative; NT, not tested (biopsy not done). †Si, stimuration index of IFN-g production by PBMCs in reseponse to HBcAg; ¶PI, proliferation index of PBMCs in response to HBcAg.

Table.3 IFN-γ production and proliferation of PBMC in response to ConA and HBcAg.

#Group	†SI		††PI	
	ConA	HBcAg	ConA	HBcAg
HBV-positive NBNC CLD (13)	27.9±4.8	8.7±1.9	3.1±0.3	1.6±0.1
HBV-negative NBNC CLD (4)	46.3±13.6	1.5±0.2	3,5±0.9	1.2±0.2
CH-B (12)	15.8±5.1	8.9±1.1	3.1±0.5	2.2±0.2
CH-C (16)	36.9±8.6	1.4±0.1	3.0±0.4	1.0±0.0
Control (33)	49.4±5.2	1.5±0.3	3.9±0.2	1.1±0.2

Data are shown as mean±SE. †SI, stimulation index of IFN-γ production; ††PI, proliferation index. ConA, concanavalin A. *:p<0.05 , **:p<0.01, *** p<0.001, **** p<0.0001.

Table 4. Relationship between serum anti-HBs and/or anti-HBc and hepatitis activity in the liver, and PBMCs response to HBcAg in HBV-positive NBNC CLD.

Anti-HBs and/or anti-HBc	Patients' age (Yr)	§HAI score	†SI	¶PI
Positive (n=5)	57.3±5.6	3.4±0.4 (n=4)	4.5±1.4	1.4±0.1
Negative (n=8)	56.7±5.9	2.6±0.9 (n=7)	5.6±1.7	1.4±0.1

Data are shown as mean±SE. §HAI score, histological activity index score category I to III. †Si, stimulation index of IFN-γ production by PBMCs in reseponse to HBcAg; ¶PI, proliferation index of PBMCs in response to HBcAg.

Figure legends

Figure. 1 Core promoter sequence of HBV DNA amplified in sera from patients with NBNC CLD.

DNA sequence of the core promoter region (upper panel) and amino acid sequence of the distal part of the X protein (lower panel) are shown. The mutant HBV infecting NBNC CLD patients showed an eight-nucleotide (8-nt) deletion. This mutation creates a stop codon downstream, and truncates the X protein by 20 amino acids (AA) (7,9). The nucleotide number of HBV DNA was based on Kanai's report (42). AT rich regions are indicated by *bars*.

Figure.2 Immunostaining of HBsAg and HBcAg in the liver of NBNC CLD.

HBsAg was seen focally in the liver of patients with HBV-positive NBNC CLD with a weak intensity (a, arrow). HBcAg was seen in the nucleus of a hepatocyte (b, arrow). Distribution of HBcAg was only scattered through the lobules. Immunoperoxy method (x100).

Figure.3 Correlation between immune response of PBMCs to HBcAg and histological activity of hepatitis in HBV-positive NBNC CLD and CH-B.

The stimulation index of IFN-γ and the proliferation index of PBMCs were significantly correlated to the histological activity of hepatitis in both HBV-positive NBNC CLD (NBNC) and CH-B. Patients with HBV-negative NBNC CLD (open circle) were not included in the correlation ansisis. HAI; histological activity index score category I to III.

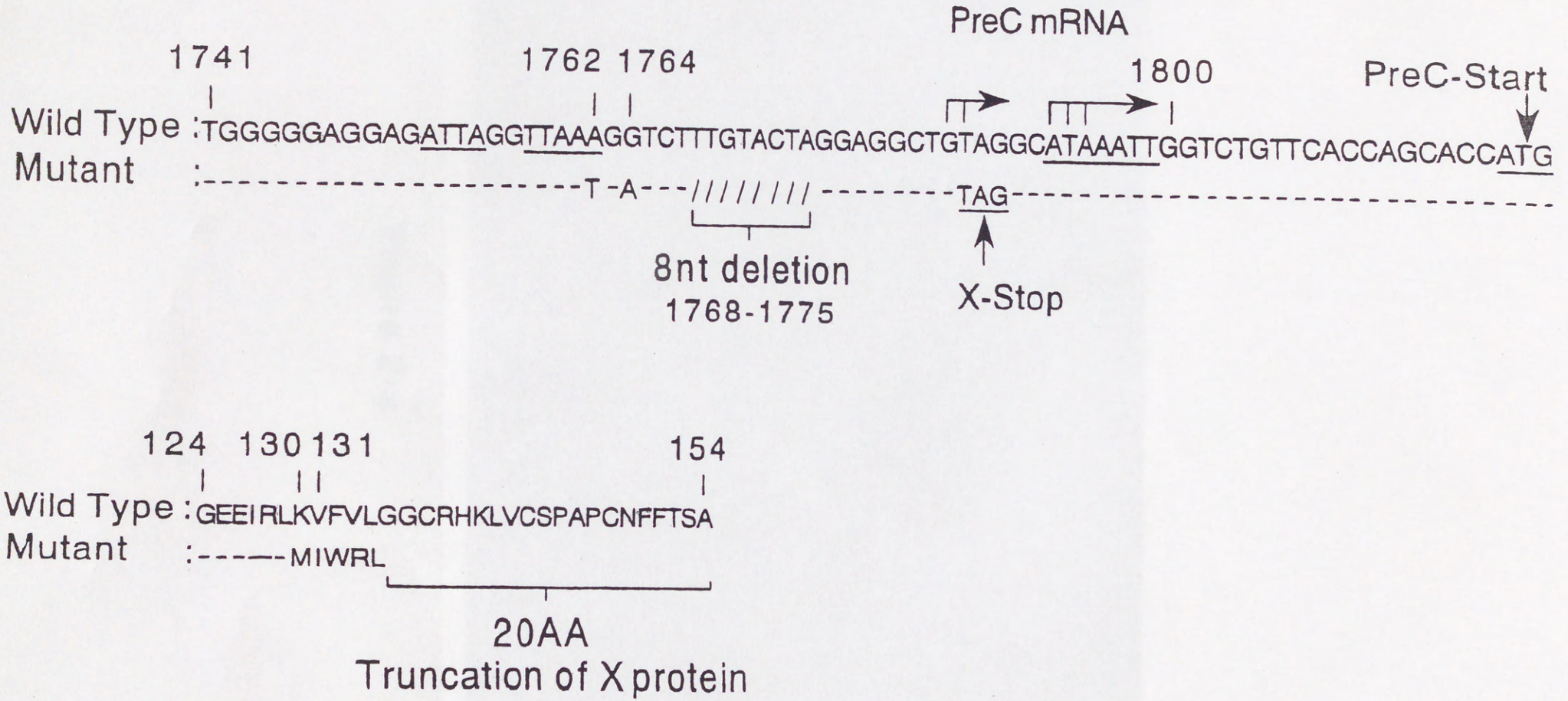

PreC mRNA
1741
1762 1764
1800
PreC-Start
Wild Type :TGGGGGAGGAGATTAGGTTAAAGGTCTTTGTACTAGGAGGCTGTAGGCATAAATTGGTCTGTTCACCAGCACCATG
Mutant :----------T-A---////////--------TAG----------
8nt deletion
1768-1775
X-Stop
124 130 131
154
Wild Type :GEEIRLKVFVLGGCRHKLVCSPAPCNFFTSA
Mutant :-----MIWRL
20AA
Truncation of X protein

Figure 1

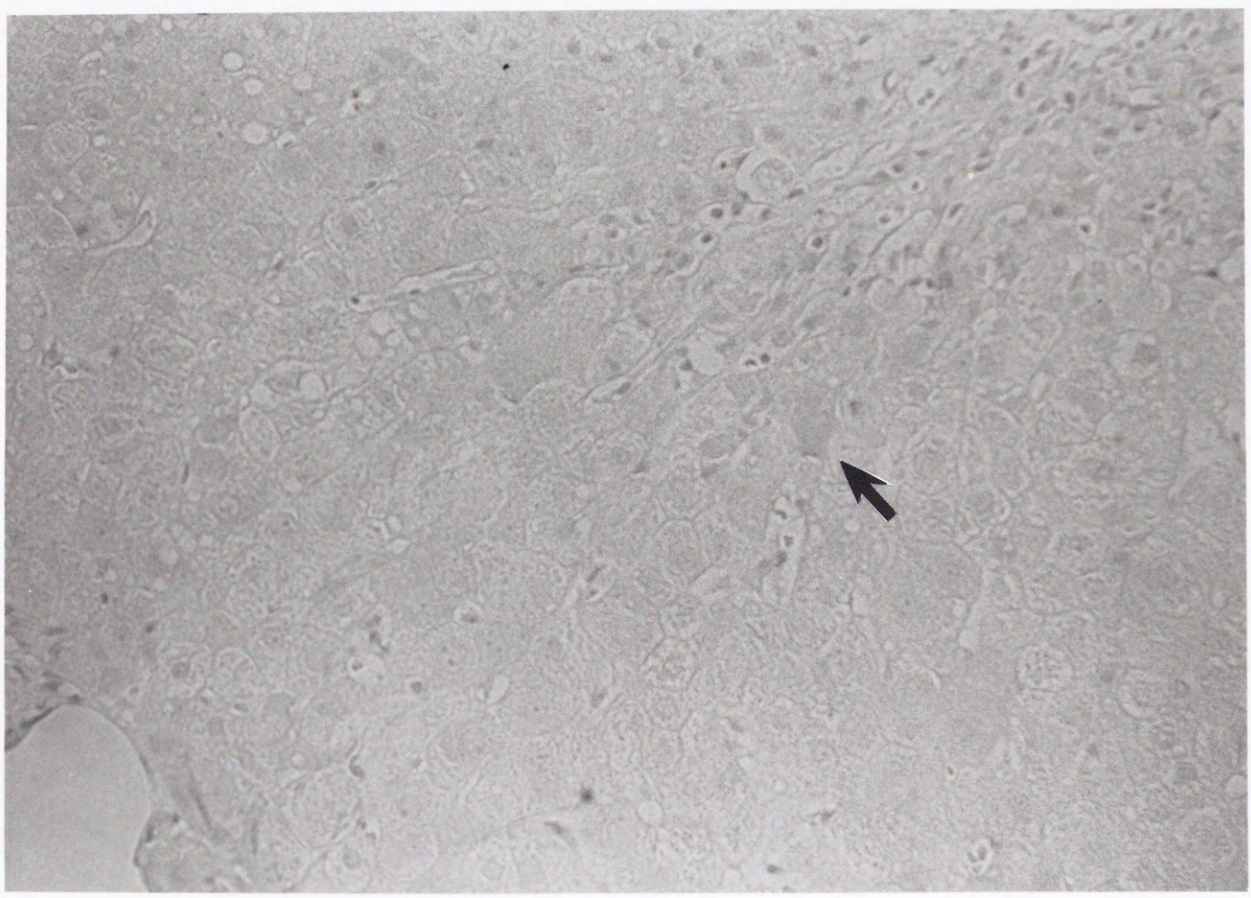


Figure 2-a

Figure 2-b

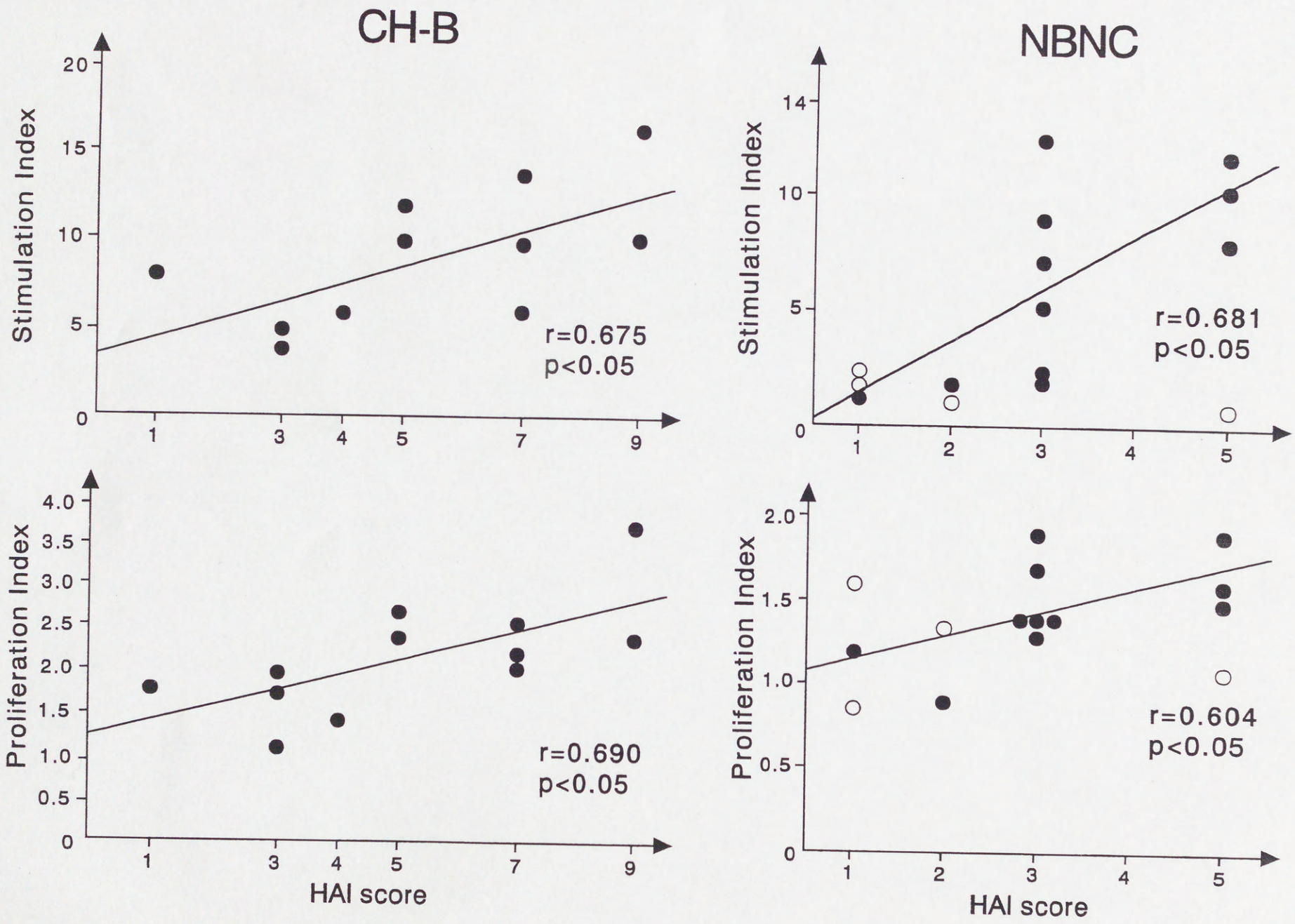
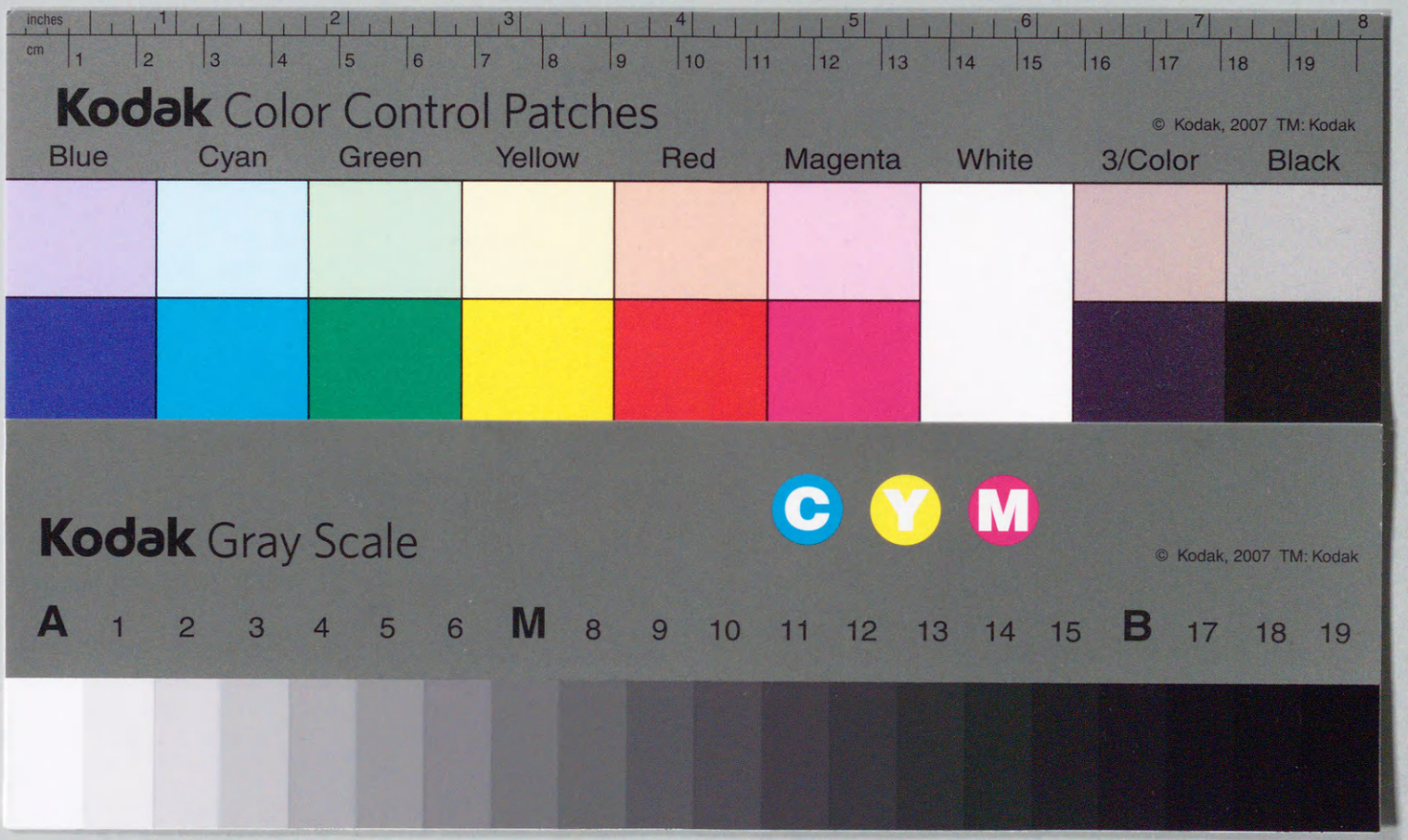

CH-B
NBNC
Stimulation Index
Proliferation Index
HAI score
r=0.675
p<0.05
r=0.681
p<0.05
r=0.690
p<0.05
r=0.604
p<0.05

Figure 3


inches
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Kodak Color Control Patches
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Cyan
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Yellow
Red
Magenta
White
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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19