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Detection of *c-MYC* Gene in Micronucleated Hepatocytes from Regenerative Cirrhotic Nodules and Hepatocellular Carcinoma of Hepatitis C Virus Infected Patients

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Abstract

c-*MYC* gene alteration has already been shown in chronic liver diseases, which includes hepatocellular carcinoma (HCC). The spontaneous formation of micronucleated hepatocytes (MN-Heps) in liver cirrhosis (LC) tissue from patients infected by hepatitis C virus (HCV), has been previously reported. The objective of this study was to investigate if the *c-MYC* gene sequence is lost by MN-Heps in cirrhotic process with or without HCC. For this purpose the presence of c-*MYC* gene in MN-Heps was investigated by fluorescence in situ hybridization in paraffin-embedded liver tissue. Five control liver samples of healthy organ donors were included in this study. Increased c-*MYC* gene copies were detected in 48-66% of MN-Heps in cirrhotic nodules from all cases, but not in control liver cells. Furthermore, gain of c-*MYC* gene number of copies was detected in 28% of the hepatocytes from regenerative and macroregenerative nodules (RNs, MRNs). The increase was also determined in 46% of tumor cells. No significant difference in extrusion of the c-*MYC* gene by RN or MRN hepatocytes was observed. We concluded that RNs and MRNs show cytogenetic abnormality towards the c-*MYC* gene. Its extrusion in MN-Heps seems to be an early event in regenerative cirrhotic lesions. The increase of c-*MYC* gene copies in LC from HCV infected patients might contribute to the development of HCC.

Keywords: *c-MYC* gene; Micronucleated hepatocytes; Liver cirrhosis; Hepatocellular carcinoma; Hepatitis C virus

Abbreviations: Hepatocellular Carcinoma (HCC); Hepatitis C Virus (HCV); Micronucleated Hepatocytes (MN-Heps); Liver Cirrhosis (LC); Fluorescence in Situ Hybridization (FISH); Regenerative Nodules (RNs); Macroregenerative Nodules (MRNs); Tumor Nodules (TNs); Normal Parenchyma (NP); Chronic Hepatitis (CH); Double Minutes (DMs)

Introduction

It is a well established fact that hepatitis C virus (HCV) is a risk factor for the development of liver cirrhosis (LC) and hepatocellular carcinoma (HCC). This type of tumor is the 7th cause of death in the world [1]. Approximately 70% of the patients that contract acute HCV infection develop chronic hepatitis (CH). Persistent HCV infection leads to chronic inflammation with destruction of hepatocytes, and an irregular regeneration of the cells. As a result of the chronic viral infection, proliferating liver cells may become more susceptible to acquire chromosomal instability and genetic alterations, a condition known as hypercarcinogenic state [2]. Chromosome instability is one of the first events observed in HCC [3], although cytogenetic alterations can already be seen by micronucleus (MN) formation in hepatocytes of RNs and MRNs from cirrhotic patients infected or not by HCV [4,5].

Micronuclei are structures that arise from acentric fragments, or whole chromosomes, that are not included in the main nucleus during cell division [6]. Fluorescence *in situ* hybridization (FISH) has been used as a powerful tool for the identification of the MN content [7,8], which may be associated with the cytogenetic abnormalities observed during carcinogenesis [9-11]. Loss of chromosomes by MN has proved to be a good indicator of response to the action of mutagens and carcinogens, as seen in hepatoma HepG2 cells [12,13].

Imbalances of the oncogene c-*MYC*, which is considered a central element in the complexity of the tumorigenic process, has been investigated during the development of tumor in several human cancers [14-17], including HCC [18-21]. The amplification of *c-MYC* gene in HCC was also described by other authors [22-25], although the specific extrusion of its sequence by MN in LC, as well as during human hepatocarcinogenesis has not been described. The loss of c-*MYC* amplified sequence by MN, showed that this mechanism can be useful to moderate tumor growth [26], and it can be used as a therapeutic strategy [27].

The main objective of this study is to detect loss of c-*MYC* gene sequence by MN-Heps from regenerative, macroregenerative, and tumoral nodules (RNs, MRNs, and TNs). The investigation was accomplished in LC cases associated or not with HCC. The comparison among the frequency of that event in all nodules was made in relation to normal parenchyma (NP).

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Material and Methods

Patient selection

Liver samples of patients with degree IV, moderately active LC, were provided by Laboratório de Patologia Diagnóstika, and Departamento de Patologia, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brasil. Samples were separated into three groups: I, II, and III. Each group comprises of five cases. These were classified as follows: I (men and women, 30-59 years old) with LC/HCV; II (men and women, 30-66 years old) with LC/HCV associated with HCC, and III (men and women 20-51 years old) healthy individuals (liver donors) as control group. All patients were evaluated serologically for HCV and HBV infections. Patient exposed to other ethiologic agent, mutagens for instance, were not included in this study.

Procedures

All samples of liver tissue were fixed in formalin (10%), and embedded in paraffin blocks. Slices 4 μm thick were placed onto slides previously treated with 3-aminopropriltrietoxiilano (Sigma USA). Selection of study areas was based on histopathological analysis of lesions stained with hematoxylin-eosin as reference. Slices were examined for the presence of RNs, and MRNs. Nodules were classified into categories, according to morphological and histological criteria proposed by the International Working Party [28]. Diagnosis of HCC grading was done according to the standard histological criteria [29].

Detection of c-*MYC* gene by FISH

The detection of gain or loss of c-*MYC* gene by MN-Heps from RNs, MNRs, and TNs from LC, associated or not with HCC, were performed in paraffin-embedded tissue sections. Paraffin was removed as described by Almeida et al. [4], in order to accomplish fluorescence in situ hybridization (FISH) assay. The locus-specific probe (LSI) c-MYC (32-19006) labeled with Spectrum Orange (Vysis Inc., Downers Grove, IL, USA), was then applied to the samples according to the manufacturer protocol. Nuclei and micronuclei were identified based on the absence or presence of fluorescence signal. Loss of c-MYC gene by MN corresponds to MN +c-*MYC* in photographs. Labeling was considered positive when more than 70% of hybridization was observed. For each nodular area (RNs, MRNs and TNs) frequency of MN in 2,000 interphase mononucleated hepatocytes (Mono-Heps) was assessed. This gives a total number of 20,000 cells (group I), 30,000 cells (group II), and 10,000 cells (group III). Nuclear gain of c-*MYC* gene copies was surveyed in 100 Mono-Heps of the same area. Analysis of +c-*MYC* gene in nuclei and MN was done under an epifluorescence Zeiss III light microscope equipped with UV epi-illumination, triple filter (DAPI, FITC and Texas Red) and immersion objective (100X). Fluorescence was evaluated after 475-495 nm excitation and 520 nm filtering. Kodak Gold 100 ASA film was employed for image documentation.

Statistical analysis

Data were subjected to statistical analysis by using both commercial (SPSS, Inc. Chicago, IL: USA) and public domain software [30]. They were considered statistically significant when p was ≤ 0.05.

Results

Detection of *c-MYC* gene in MN-Heps

Hybridization of Mono-Heps of all paraffin-embedded liver tissue samples from groups I, II and III with fluorescent c-*MYC* probe was at least 70% efficient. The extrusion +*c-MYC* gene by MN-Heps from nodules occurred in all cases of cirrhosis, and in those related to HCC (groups I and II). It was not observed in MN-Heps from the control group (III).

The vast majority of MN-Heps from nodules of groups I and II, shows a +*c-MYC* gene double signal in MN. This does not depend on its size and signal number of this gene seen on the main nucleus (Figures 1a and 1e). Few MN-Heps of cases from group II displaying a large number of +c-*MYC* signals in their nuclei, have more than two copies of this gene per MN (Figure 1c).

MN-Heps with two or more +c-*MYC* signals in the main nucleus, may not have in MN (Figures 1b and 1d). In MN-Heps from MRNs, more than two +c-*MYC* signals were found. MN of different sizes have or not the +c-*MYC* signal (Figure 1e). In RNs and TNs, some MN-Heps have two MN with only one signal in both main nucleus, and MN (Figure 1f).

In group I, the range of +c-*MYC* gene in MN-Heps was 5-18 (11.4 ± 5.12) for RNs and 11-18 (14.6 ± 2.7) for MRNs. In group II, the values ranged from 6-26 (12.4 ± 8.44) for RNs, 9-35 (15.4 ± 11.01) for MRNs, and 10-31 (20 ± 7.51) for TNs. In group III (control), the few MN-Heps detected, did not show signals of the +c-*MYC* gene.

Comparison of the frequencies of +c-*MYC* gene in MN-Heps from RNs, MRNs from groups I and II, and the TNs (group II) showed that they were higher and significant in relation to control group ($p<0.05$). This fact was also evident when compared with frequencies in TNs and

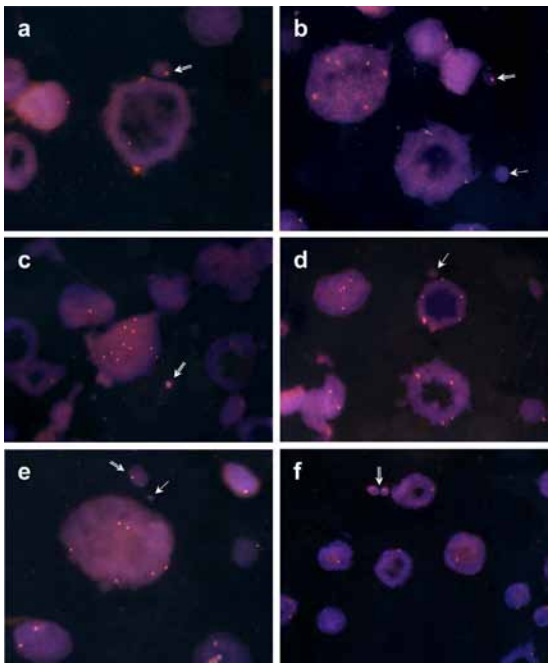

Figure 1: FISH analysis of interphase nuclei with MN using LSI probe (orange) for c-*MYC* gene. Photomicrographs of loss c-*MYC* gene by MN-Heps detected in samples from cirrhotic and HCC nodules of HCV infected patients. **a**- MN with one signal; **b**- binucleated hepatocytes with one signal in the nucleus, and another in MN; **c**- hepatocyte with ten signals in the nuclei and one in MN; **d**- hepatocyte showing a large MN without signal and nucleus having four signals; **e**- hepatocyte with two MN, with and without signal; **f**- hepatocyte with two MN, each one with one signal, and also in the nucleus. The arrows indicate: (→) MN without signal, and (⇒) MN with +c-*MYC* signal.

RNs from group I. Comparison of +c-*MYC* gene frequency between RNs (I) and MRNs (II) in MN-Heps, showed no significant differences (Table 1). Furthermore, the comparison between RNs and MRNs (I and II) also showed no significant difference among themselves (Table 1). Similarly, comparison between TNs and RNs (II), and with those of MRNs (II) were not different, (Table 1).

As is shown in Table 1, all scored groups have c-*MYC* gene losses ($p< 0.05$) when compared to NP. The internal comparison among these groups indicates a similar variation of the +c-*MYC* gene ($p\sim1.000$)

The increased number of +c-*MYC* signals in nuclei was detected in 28% of the Mono-Heps from RNs and MRNs, and 46% of TNs of all cases studied. In RNs from groups I and II, hepatocytes with +c-*MYC* were observed, ranging from 3-5 signals per nucleus in more than 10% of Mono-Heps. This was also evident in MRNs and TNs, but in a higher frequency. In the same area, some nuclei showed 6 to 9 +c-*MYC* gene signals (Figure 2), and a small proportion of hepatocytes with more than 9 copies of the gene. In the control samples, there was no increased number of +c-*MYC* signals, 98.6% of liver nuclei displaying two +c-MYC gene signals (Figure 2). The results of this analysis are shown in Table 2.

Discussion

For the first time, we identify signals of copies of c-*MYC* gene lost by MN-Heps from cirrhotic liver samples of patients infected by HCV, with or without HCC. A retrospective analysis was efficient and enabled us to obtain an evaluation of MN-Heps of archived samples. Using interphase cytogenetic FISH method, we evaluated the frequency of elimination of c-*MYC* gene by MN-Heps from ordinary RNs and MRNs, and TNs respect to NP. We verified that the frequency of elimination of c-*MYC* gene by MN-Heps from RNs and MRNs of the cirrhotic groups (I and II) was not different among them ($P>0.05$), suggesting that this type of cytogenetic changes, occurs in early stages of cirrhotic process and, it does not depend on the presence of HCC. In our previous data, we had already considered the ordinary cirrhotic nodules (RNs and MRNs) cytogenetically abnormal as evaluated by MN formation [4].

The frequency of c-*MYC* gene elimination by MN-Heps from TNs is not different from that detected in RNs from LC cases, and also in relation to RNs, and MRNs from LC/HCC associated cases (Table 1). These frequencies of loss of c-*MYC* gene by MN-Heps, probably should be related to the chronic HCV infection that leads to cell death, irregular regeneration, or chromatin degradation by nucleases from MN [26].

The elimination of c-*MYC* gene by MN-Heps, persistently present in RNs, MRNs, and TNs did not occur in healthy liver tissue. These differences might be related to chronic HCV infection, since the proliferation of hepatocytes in regenerative response to any injury, is an important event, especially in cirrhotic livers of patients infected with HCV [31,32], predisposing to the formation of MN. In the recent past, the size of MN has been considered as an indicator of the extent of chromosomal changes [6,33]. The long period of inflammation leading to complex cirrhosis process, may have contributed to the accumulation of genetic damage, justifying the formation of different MN sizes.

In our study, we detected MN-Heps with c-*MYC* gene represented by one or two copies of signals of this gene, and other cells with one signal in MN, and another in the main nucleus, in addition to hepatocytes with a couple of signals in the nucleus, and another in MN. These events may result from chromosomal rearrangements (as isochromosomes), and aneuploidy induced by deregulation of c-*MYC* gene [34]. Increased number of copies of the c-*MYC* gene has been reported in the hepatocyte nuclei of HCC samples [20,21,24,25], as well as in other types of cancer, and the amplification of c-*MYC* gene has been associated with advanced stages of tumor development [14,15,17]. In the present study, we also found increased signals of c-*MYC* gene (up to 9), not only in hepatocytes of HCC, but also of RNs and MRNs, considered as benign lesions, a histopathogical point of view. In a rare study, evaluating c-*MYC* gene amplification in multiple steps of breast cancer evolution, the authors did not found amplification, neither in hiperplasia, nor in normal adjacent cells. So it appears that c-*MYC* gene amplification is a late event, being detected only in invasive cells, in the breast cancer progression [35]. In all RNs and MRNs, we found increased signals of c-MYC gene, and we speculated on the possibility that the overexpression of c-MYC protein found in the same samples of RNs and MRNs (unpublished data), may be related to the alteration of

Groups (area)	NP (II)	RN (I)	MRN (I)	RN (II)	MRN (II)
RN(I)	<0.0001	-	-	-	-
MRN(I)	<0.0001	0.9856	-	-	-
RN(II)	<0.0001	0.9999	0.9998	-	-
MRN(II)	<0.0001	0.9999	1.0000	1.0000	-
TN(II)	<0.0001	0.9998	0.9998	0.9998	0.9009

Significance set at P<0.05

Table 1: *P* values calculated from multiple comparison of loss of *c-MYC* gene by MN-Heps on regenerative (RN), macroregenerative (MRN) and tumoral (TN) nodules from LC/HCV without (I) and with HCC (II) groups in relation to control group (III) - normal parenchyma (NP).

			% of Mono-Heps *c-MYC* gene copies number		
Groups (area)	**Mono-Heps**	**0-2**	**3-5**	**6-9**	**>9**
RN (I)	100	71.2±5.58	13.6±4.21	10.0±3.74	5.2±1.92
MRN (I)	100	70.6±5.07	12.4±4.03	12.4±2.07	5.4±4.15
RN (II)	100	74.4±9.31	12.6±3.97	10.2±6.67	4.8±5.89
MRN (II)	100	74.4±9.31	13.0±2.12	12.4±6.69	5.6±4.33
TN (II)	100	57.8±7.32	21.0±7.68	18.0±4.94	7.2±4.49
NP (III)	100	98.6±1.94	1.0±1.41	-	-

Table 2: Distribution of *c-MYC* gene copies number in mononucleated hepatocytes (Mono-Heps) of regenerative (RN), macroregenerative (MRN) and tumoral (TN) nodules from LC/HCV, without (I) and with (II) HCC cases, and control group (III) - normal parenchyma (NP).

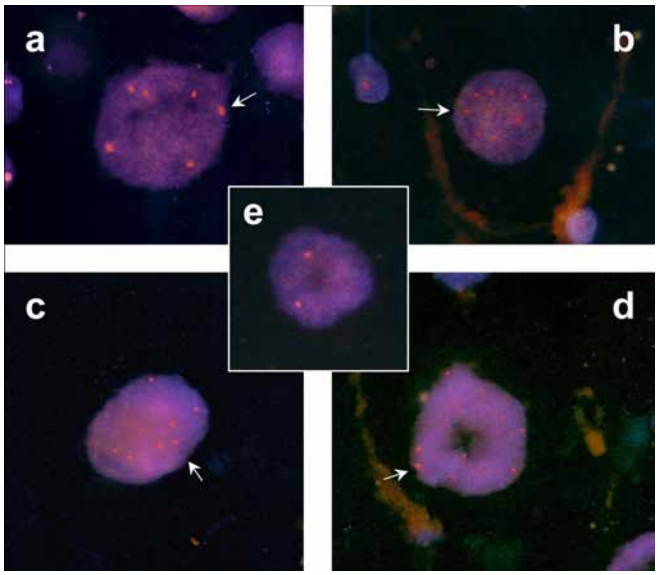

Figure 2: FISH analysis of interphase nuclei using LSI probe (orange) for *c-MYC* gene. Photomicrographs of hepatocyte nuclei from cirrhotic and HCC nodules of HCV infected patients. **a, b, c, d**: hepatocyte nuclei from LC with or without HCC showing 6, 7, 7 and 10 signals; **e**: hepatocyte nucleus from NP showing two signals. The arrow (→) indicates +c-*MYC* signal.

number of copies of c-*MYC* gene in these nodules in relation to normal parenchyma. During the hepatitis viruses, chromosomal instability is a common event mediated by recombinogenic proteins [2]. According to other authors [34,36,37], c-MYC protein has been considered a structural modifier of the genome that affects nuclear organization. Deregulation of the expression of its protein can mediate genomic instability, initiating both, intra- and extra-chromosomal amplification mechanisms.

Based on the patterns of signals of c-*MYC* gene found in hepatocytes, independent of cirrhotic group (with and without HCC), in this study, the high number of amplified signals of the *c-MYC* gene in MN or in the main nucleus are represented by double minutes (DMs). Since DMs are autonomous structures that do not have centromeres, and have a random distribution in the nucleus, they might provide their expulsion of hepatocyte nuclei. These extrachromosomal elements can be segregated in a stable form to daughter cells during mitosis, but the mechanism by which DMs are stick to the chromosome arm is not known. In addition, DM-MN type can be generated, both at mitosis and interphase of the cell cycle [8,33,38,39]. These authors suggest that the amplified DNA can be reduced by *in situ* degradation or elimination by MN. The excess of amplified DNA, by an active process, concentrates on the peripheral of the nucleus, and it may be expulsed by MN in a bud formation mechanism. The MN is extruded from the cytoplasm, during S phase of the cell cycle. We believe that such mechanisms might occur, also in hepatocytes. Nevertheless, none of the mechanisms of extrusion of amplified gene are well understood.

Similar events were shown in blast cells from patients with acute myeloid leukemia expulsion of clusters of signals of amplified c-*MYC* gene by MN, probably as DM [17]. Also, the induced or spontaneous elimination of amplified c-*MYC* gene by MN in Colo320DM and HL60 cell lines, suggests a strategy to reduce the tumorigenic potential and reversion of the malignant phenotype [26,40].

Our data are not sufficient to determine, with accuracy, the mechanisms by which c-*MYC* sequences were lost by MN-Heps. Nevertheless, we believe that in cirrhotic process, the formation of the MN as chromosome-type and/or DM-type by genetic and/or epigenetic mechanisms [39] is a likely process.

Considering the role of c-*MYC* in human hepatocarcinogenesis [41], it is important to note that no data are forthcoming about the elimination of this sequence (amplified or not), by MN and their fate in the liver tissue. From what we know, the MN is commonly observed in cancer, and it is considered an indicator of DNA damage, representing genetic instability, that contributes to the risk of development of degenerative diseases [8]. As the amplification of c-*MYC* has been related to the degree of aggressiveness in HCC [18,20,42], it is possible that the expulsion of that sequence by spontaneous MN formation, may have implications for the clinical prognosis of the patient. Cytogenetic and molecular mechanisms in the cirrhosis development have not yet been elucidated, so the importance of nuclear loss of a specific gene such as c-*MYC*, by MN, needs further studies to establish its prognostic impact.

Our data showed for the first time that, loss of c-*MYC* gene by MN-Heps, is an early event in hepatocarcinogenesis. Evaluation of c-*MYC* gene loss by MN-Heps associated to FISH technique has been proven to be useful to identify the type of cytogenetic instability in archived samples of cirrhotic liver tissue, and to estimate the potential risk of development of HCC.

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