Regulation of telomere length and telomerase activity in dysplastic nodule of human multistep hepatocarcinogenesis

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Regulation of telomere length and telomerase activity in dysplastic nodule of human multistep hepatocarcinogenesis

Directed by Professor Young Nyun Park

The Master's Thesis submitted to the Department of Medical Science, the Graduate School of Yonsei University in Partial fulfillment of the requirements for the degree of Master of Medical Science

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This certifies that the Master's Thesis of Kwang Jo Chae is approved.

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참 어렵게 오랜 시간이 지나서야 마무리를 짓게 되었습니다. 힘들고 어려운 시간들이 이어져서 감회가 새롭습니다. 못난 아들 믿고 기다려 주시고 제가 무엇을 하던지 한없는 신뢰를 보내주셔서 감히 포기라는 단어를 생각지도 못하게 해주시는 부모님께 먼저 감사를 드리고 시련을 던져주셔서 저를 단련시켜주신 하나님께 감사 드립니다. 그리고 제게 기회를 주셔서 소중한 경험을 하게 해 주시고 모자란 제자 때문에 신경 많이 쓰셨을 박영년 선생님, 새로 만들어진 실험실에서 아무것도 모르는 제게 research 가 무엇이라는 것을 가르쳐주신 오봉경 선생님, 마무리에 정말 많은 도움을 주신 박찬일 선생님과 이관식 선생님, 그리고 제가 졸업 할 수 있도록 마음 써주신 김호근 선생님께 송구하고 감사 드립니다. 해 준 것도 없는데 먼저 졸업해서 미안하다는 내 동생 선애에게도 고마운 마음 가득합니다. 대학원 생활을 하면서 마음의 힘이 되어준 연락이, 민정이, 과 후배로 같은 실험실에 들어와 잘 따라준 영주, 찬희, 옆 실험실에서 활력이 되어 주었던 윤석이와 연정이 숙희 에게도 고맙다는 말을 전합니다.
언제나 힘이 되어주는 고마운 친구 윤창이, 남규, 병호, 승규, 경순, 은영이, 졸업도 하기 전에 저를 믿고 뽑아주신 서울대 김현정 선생님과 한중석 선생님께 감사 드리고 사회생활의 시작을 좋은 곳에서 시작할 수 있도록 도와준 한국로슈진단도 제게는 고마운 존재 입니다. 제가 깔끔하게 마무리 짓지 못한 것을 마무리 지을 수 있도록 마음 써주신 이순신 이사님과 김병오 부장님, 학교와는 다른 사회생활을 나름대로 신나고 재미나게 할 수 있도록 도와준 동규와 상백이 그리고 늦게 시작한 사회생활에 도움이 되는 말씀해 주신 우리 학술부 선배님들께 참 많이 고맙습니다. 고마운 분들이 너무 많아서 이 한 장의 종이가 부족하지만 모든 분들께 감사 드립니다.
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ABSTRACT

Regulation of telomere length and telomerase activity in dysplastic nodule of human multistep hepatocarcinogenesis

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(Directed by Professor **Young Nyun Park**)

Aim : There is Increasing evidence of human mutistep carcinogenesis and dysplastic nodules (DNs) are considered as a preneoplastic lesion. Telomere shortening and telomerase reactivation is an important early event of hepatocarcinogenesis; however their regulation mechanism is still unknown. For that reason, we describe that the maintenance of telomere with telomerase reactivation, was studied in hepatocarcinogenesis for the characterization of borderline lesions.

Materials and Methods : Three chronic hepatitis (CH), 10 cirrhosis, 7 large regenerative nodules (LRNs), 30 low grade dysplastic nodules (LGDNs), 6 high grade dysplastic nodules (HGDNs), 3 DNs with hepatocellularcarcinoma (HCC) foci, 11

HCCs, and 4 normal livers were included in the study. The terminal restriction fragment length (TRFL) and telomerase activity (TA) were examined by Southern hybridization and TRAPeze Elisa telomerase detection kit.

Result : The TRFL and TA showed significant differences between the LGDNs and HGDNs. Most LGDNs had similar levels of TRFL and TA to those of the CH, cirrhosis and LRNs, however, 17% of LGDNs revealed shortening of telomeres up to the levels of HGDNs and 7% of LGDNs showed high levels of TA. The levels of TRFL and TA in HGDNs showed no significant differences from those of DNs with HCC foci and HCCs.

Conclusion : Telomere shortening and telomerase reactivation occur particularly in the course of transition from chronic liver disease and LGDNs to HGDNs, but not from HGDNs to HCCs. Accordingly, the changes of telomerase length and TA might occur in the early stage of hepatocarcinogenesis.

Keywords: Telomere, Telomerase, Dysplastic nodule, Hepatocellularcarcinoma, Large regenerative nodule, Hepatocarcinogenesis, Cirrhosis

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Ⅰ. Introduction

Telomeres located at the end of the chromosomes are composed of tandem arrays of a short DNA sequence, i.e. d(TTAGGG)n in vertebrates [1]. Their multiple functions, including stabilizing the natural end of the chromosome and protecting it from end-to-end fusion, are crucial to cell viability. Telomerase, which synthesizes the telomeric DNA, is involved in both maintaining and regulating the telomere length [2]. The end-replication problem, or the inability of DNA polymerases to complete replication of the end of a DNA duplex [3], results in a shortening of the telomere in proportion to each cell replication [4].

The maintenance of the telomeres, together with the activation of telomerase, may be an obligatory step in the development of most tumors. Contrary to normal cells, immortalized cells in cultures and cancer cells in vivo exhibit a short but stable telomere length, which is maintained by the action of the telomerase.

Recently, there has been increasing evidence concerning human multistep hepatocarcinogenesis emphasizing the preneoplastic nature of the large nodules, which are referred to as DNs or adenomatous hyperplasia [5,6]. However, the decision where to draw the diagnostic line between dysplasia (not yet a HCC) and a well differentiated HCC is often difficult. The histological differentiation of low grade DNs from the large regenerative nodules (LRNs) in cirrhosis is not easy, although vascular changes such as unpaired arteries and sinusoidal capillarization are helpful [7,8], and the pre-malignant nature of low grade DNs appears less resolved than that of high grade DNs. This borderline category is currently an area of controversy, which leads to confusion when trying to compare studies from different investigators. Telomere shortening and telomerase reactivation has been reported in the development of HCC from chronic liver disease, however, these changes in low and high grade DNs are still not clear [9-12].To characterize the borderline lesions of human multistep hepatocarcinogenesis, the

telomere length and the levels of TA were studied in chronic hepatitis (CH), cirrhosis, LRNs, low grade DNs, high grade DNs, DNs with HCC foci and HCCs.

Ⅱ. Material and Methods

1. Tissue samples

The nodular lesion was subdivided into 5 groups as follows; 1) large regenerative nodule (LRN), 2) low grade DN, 3) high grade DN, 4) DN with HCC foci, and 5) HCC according to the standard criteria of an international working party (Figure 1).

We studied terminal restriction fragment (TRF) length and telomerase activity (TA) in 7 large regenerative nodules (LRNs), 30 low grade dysplastic nodules (LGDNs), 6 high grade DNs (HGDNs), 3 DNs with hepatocellular carcinoma (HCC) foci, 11 HCCs, 3 chronic hepatitis (CH), and 10 cirrhosis of 13 patients (male: female= 10:3, mean age SD= 47.5±7.51 years). All patients had hepatitis B virus related chronic liver disease.

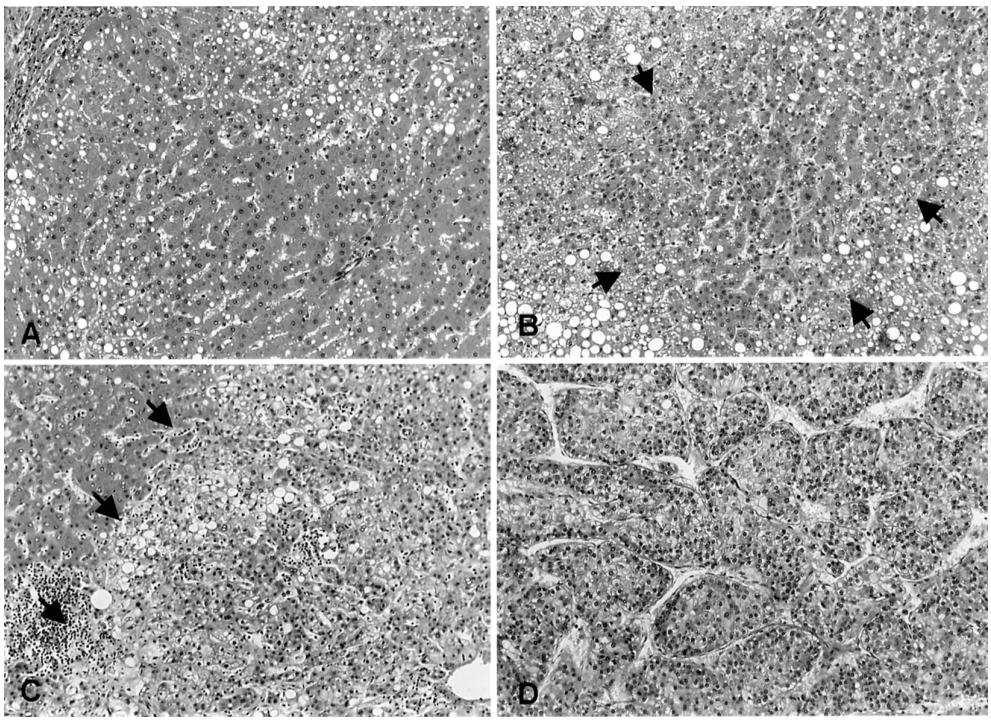

Figure 1. Histological features of (A) low grade dysplastic nodule, (B) high grade dysplastic nodule with small liver cell dysplasia (arrows), (C) dysplastic nodule with hepatocellular carcinoma foci (arrows), (D) hepatocellular carcinoma with trabecular pattern (H-E, 100×).

2. CD34 immunohistochemistry

The neoangiogenesis of an unpaired artery and a sinusoidal capillarization was evaluated in each lesion to support the diagnosis. Serial sections of each lesion were immunostained with monoclonal antibodies against alpha-smooth muscle actin (Dako, Carpenteria, CA) for the detection of the muscular layer of the unpaired artery and against CD34 (Biogenex, San Ramon, CA) to detect the sinusoidal capillarization, as previously described [7]. The numbers of unpaired arteries were counted in 10 random fields at 100 X magnification. The CD34 expression of the sinusoidal endothelial cells was evaluated as follows; grade 0, undetectable; 1 + , staining of some sinusoidal endothelial cells occupying approximately <10% of sinusoidal liver cell surface; 2 + , staining of endothelial cells occupying 10–30% of the sinusoidal liver cell surface; 3 + , staining of endothelial cells occupying 30–50% of the sinusoidal liver cell surface and 4 + , staining of endothelial cells occupying >50% of the sinusoidal liver cell surface.

For use as controls, normal liver tissues were obtained from the resected liver for benign lesions or metastatic carcinomas of four male patients with the age range from 42 to 74 years. The controls did not have the hepatitis virus, and showed relatively normal liver histology, with the exception of a mild fatty change (Figure 2, Figure 3).

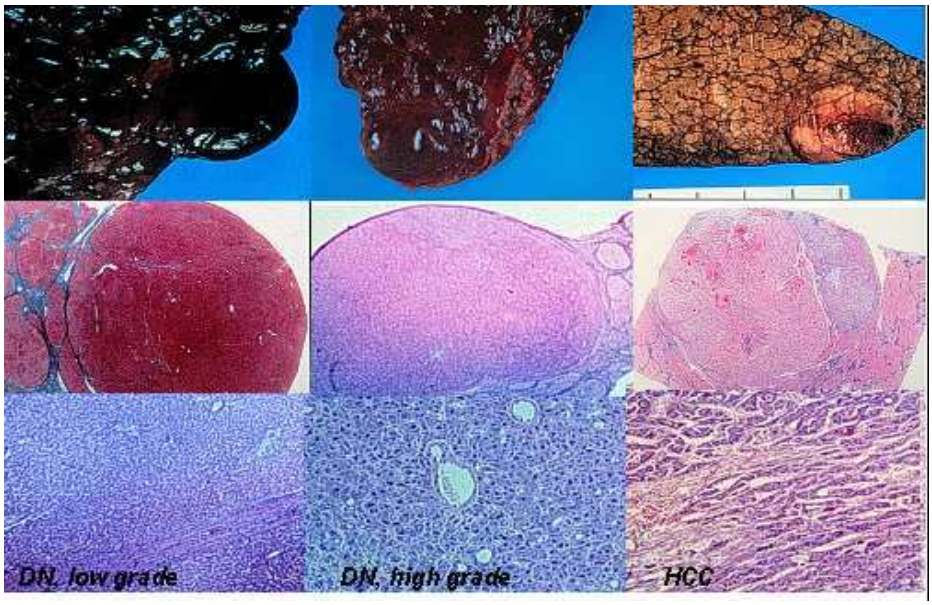

Figure 2. Histological and gross picture for DN low grade, DN high grade, and HCC. The differentiation of HCC was evaluated according to Edmondson's grading system

3. Tissue extraction

A tissue sample, wash in cold PBS buffer 2 times, and then, weighing 15–30 ㎎, was pulverized with a pestle under liquid nitrogen, and re-suspended in 200 ml of the lysis buffer (1X CHAPS buffer) provided with the kit, in which the RNase inhibitor (Ambion, TX, USA) had been added to a final concentration of 100 units/㎖ prior to extraction. The cells were transferred to microtubes and lysed using the freeze–thaw method. After centrifugation at 15,000 rpm for 30 min at 4 ℃, the protein concentration in the supernatant was measured using a protein assay kit (BioRad, CA, USA).

4. Genomic DNA extraction

The genomic DNA was extracted from the tissue sample stored at -80℃. The tissue (30㎎) were powdered by grinding thoroughly with a pestle and mortar in liquid nitrogen. The tissue powder was scraped into 2㎖ microcentrifuge tube filled with 600㎕ of extraction buffer TNES (20 mM Tris-HCL [pH 8], 400 mM NaCl, 5mM

EDTA [pH 8], 1% (v/v) SDS) containing 1㎎/㎖ of proteinase K, and 20 ㎍/㎖ of RNase A. The samples were incubated at 55℃ overnight with shaking gently to avoid aggregation. Equal volume (600㎕) of phenol:chloroform (1:1) was added to the extract, followed by shaking softly at room temperature for 30 min and centrifugation at 13,000 rpm for 5 min, and then the upper aqueous phase was transferred to a clean tube. The process was repeated twice to clear aqueous phase. One volume (600㎕) of isophenol was added to the aqueous phase, and mixed carefully by inversion. The mixture was then centrifuge at 13,000 rpm at 4℃ for 5 min. the pellet was rinsed with 70% ethanol and allowed to dry briefly in air. The DNA was finally re-suspended in 300㎕ of ddH_2O, followed by rocking gently overnight. The quantity of genomic DNA was determined by UV spectrophotometry using UV-1601 (Simadzu).

5. Telomere terminal restriction fragment (TRF) length analysis

The telomere length was measured using Southern hybridization. Ten micrograms of genomic DNA, digested with 100 units of Hinf I overnight at 37 ℃, was obtained by ethanol precipitation. The Hinf I-digested DNA concentration was re-

measured by spectrometry, and two micrograms were fractionated on a 0.7% agarose gel using electrophoresis. Typical upward capillary transfer was used to transfer the DNA from the gel to a nylon membrane. Hybridization was carried out with a 30-end DIG labeled d(TTAGGG)$_4$ (Roche Molecular Biochemicals, Mannheim, Germany) at 37 ℃ for 5–6 h. The hybrids were washed and detected as recommended by the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). The resulting X-ray film was scanned with a luminescent image analyzer (Fujifilm, Tokyo, Japan), and the telomere signal in each lane quantified in a grid object, defined as a single column with 25 rows, using the Image Gauge Software 2.54 (Fujifilm, Tokyo, Japan). The mean telomere restriction fragment (TRF) lengths calculated by a previously described method [13].

6. Telomeric repeat amplification protocol (TRAP) assay

The telomerase activity was examined by a telomeric repeat amplification protocol (TRAP) using a TRAPeze Elisa telomerase detection kit (Intergen, NY, USA). In order to determine amount of input proteins required for the assay, TRAP assays

were initially performed using 0.05–1 ㎎ of the HCC lysate. As the amount of protein was increased, the activity increased, and reached a maximum at 0.2 ㎎. The maximum activity was maintained between 0.2 and 1 mg. Therefore, all the TRAP assays were carried out with 0.2 ㎎ of lysates. The assays were performed at 30 ℃ for 30 min for the telomere extension in 4X TRAP solution [20Mm Tris-HCl (pH 8.5), 1.5mM $MgCl_2$, 63Mm KCl, 0.005% Tween 20, 1mMEGTA] added 0.2㎍ TS primer, 2.5㎍ BSA, and 1.25 mM dNTP, followed by telomere amplification by 32 cycles of two-step PCR at 94 ℃ for 30 s, and 55 ℃ for 30 sec in PCR solution included 2.5mM dNTP, 5ng NT primer, 0.1pg TSNT primer, and 2.5 unit Taq polymerase (Promega, Medison, USA). As negative controls, the lysates were heat-treated at 85 ℃ for 10 min to inactivate the telomerase prior to the TRAP assay. The activity was semi quantified using ELISA according to the manufacturer's protocol. The series of reactions were performed in triplicate or quadruplicate, and the mean value taken as the activity of the sample. As an aid to data interpretation, direct visualization of the TRAP ladder was accomplished by 12% polyacrylamide gel electrophoresis, with ethidium bromide staining.

7. Statistical analysis

Values were expressed as the mean ± standard deviation (S.D.). Statistical analyses were performed using the Chi-Square and Mann–Whitney tests, as deemed appropriate. A *P*-value <0.05 was considered as statistically significant.

Ⅲ. Results

1. Clinical and pathological findings.

Seven LRNs, 30 low grade DNs, 6 high grade DNs, 3 DNs with HCC foci, and 11 HCCs, were studied in 13 patients (male/female = 10:3, mean age ± S.D. = 47.5 ± 7.51 years) (Table 1). All patients had the hepatitis B virus related chronic liver disease, including 10 cirrhosis and three chronic hepatitis (CH). The diagnoses and size of the lesions for each patient are summarized in Table 1. Seven patients with an explanted liver were found to have multiple nodular lesions. All nodular lesions showed no or very sparse infiltration of inflammatory cells. The differentiation of the HCCs was as follows: grade I in 2, grade II in 5 and grade III in 4, and all cases of the HCC foci in the DNs were grade I. The HCC of patient number 7 exhibited total necrosis due to a pre-operative transarterial chemoembolization, so was excluded from further study.

Table 1. Clinical and pathological findings of the patients

Patient no.	Age	Sex	Pathological diagnosis of nodular lesion (cm) / background liver
1	49	M	LRN (1.0, 1.2), LGDN (1.0, 1.0, 1.0, 1.3, 1.3, 1.6), HCC (4.5)/cirrhosis
2	51	M	LRN (0.5), LGDN (0.7, 0.8, 0.9, 0.9, 1.0, 1.2)/cirrhosis
3	44	F	LGDN (1.3), HGDN (0.9, 1.2), DN with HCC foci (1.0), HCC (1.6, 3.0)/cirrhosis
4	52	M	LGDN (0.5, 0.6, 0.9), HGDN (0.8, 0.8, 0.8, 0.9), DN with HCC foci (1.2,1.5), HCC (1.3, 3.0)/cirrhosis
5	32	F	LRN (0.7), LGDN (1.0, 1,2)/cirrhosis
6	48	M	LRN (0.8,1.5), LGDN (0.7, 0.8, 1.0, 1.0, 1.2, 1.3, 1.4, 1.5,1.5, 1.5)/cirrhosis
7	48	M	LRN (0.9), LGDN (1.0, 1.0), HCC[a] (3 m)/ cirrhosis
8	56	M	HCC (4.0)/cirrhosis
9	54	F	HCC (4.0)/cirrhosis
10	40	M	HCC (11.0)/cirrhosis
11	60	M	HCC (11.0)/chronic hepatitis
12	43	M	HCC (15.0)/chronic hepatitis
13	41	M	HCC (15.0)/chronic hepatitis

Abbreviations: LRN, large regenerative nodule; LGDN, low grade dysplastic nodule; HGDN; high grade dysplastic nodule; DN, dysplastic nodule; HCC, hepatocellular carcinoma.

[a] Total necrosis due to pre-operative transarterial chemoembolization.

The neoangiogenesis of an unpaired artery and sinusoidal capillarization is summarized in Tables 2 and 3. All cases of normal liver, CH, cirrhosis and LRN showed less than six unpaired arteries per 10 X 100 fields, and they all showed no or grade 1 of sinusoidal capillarization.

Table 2. Number of unpaired arteries[a] in human multistep hepatocarcinogenesis

	0-5	6-15	16-25	26-35	>36
Normal	4	0	0	0	0
CH/cirrhosis	13	0	0	0	0
LRG	7	0	0	0	0
LRDN	9	19	2	0	0
HGDN	0	3	2	1	0
DN with HCC foci	0	0	2	1	0
HCC	0	0	0	0	11

Abbreviations: CH, chronic hepatitis; LRN, large regenerative nodule; LGDN; low grade dysplastic nodule; HGDN, high grade dysplastic nodule; DN, dysplastic nodule; HCC, hepatocellular carcinoma.

[a] Per 10 fields x 100 magnification.

Table 3. Semi-quantitative assessment of CD34-positive sinusoidal endothelial cells in human multistep hepatocarcinogenesis

	0	1+	2+	3+	4+
Normal	4	0	0	0	0
CH/cirrhosis	4	9	0	0	0
LRG	0	7	0	0	0
LRDN	0	10	17	3	0
HGDN	0	0	4	2	0
DN with HCC foci	0	0	1	2	0
HCC	0	0	0	0	11

Abbreviations: CH, chronic hepatitis; LRN, large regenerative nodule; LGDN; low grade dysplastic nodule; HGDN, high grade dysplastic nodule; DN, dysplastic nodule; HCC, hepatocellular carcinoma.

The neoangiogenesis increased significantly according to the following progression, from LRNs, to low grade DNs, to high grade DNs, to DNs with HCC foci, and finally to HCCs ($P < 0:001$) (Figure 2).

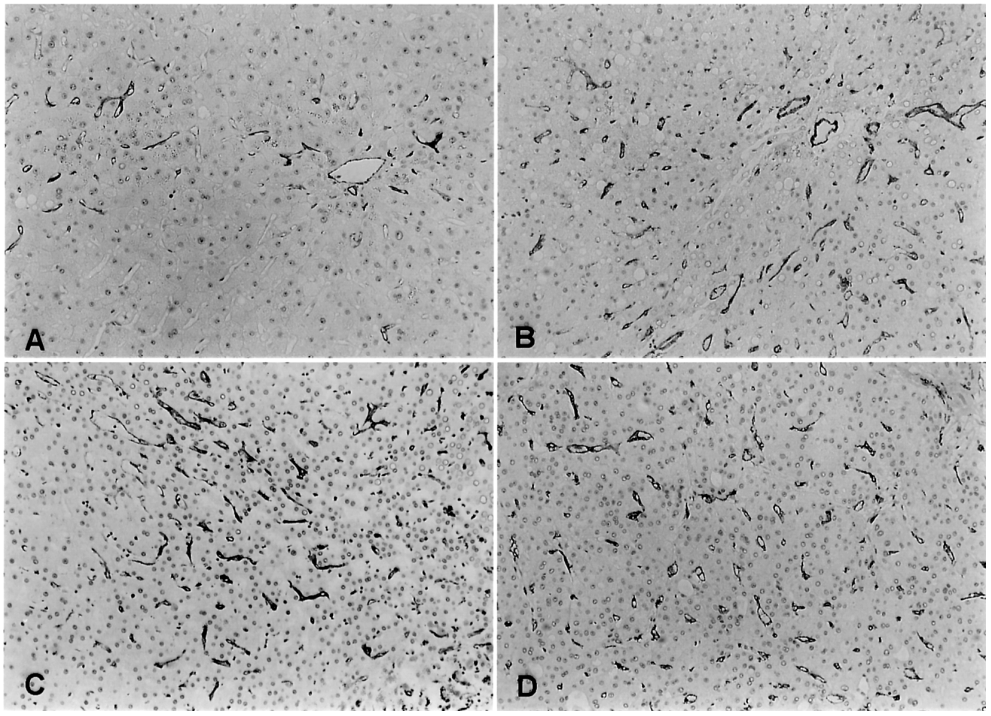

Figure 3. (A) Sinusoidal capillarization demonstrated by immunohistochemical staining for CD34 in low grade dysplastic nodules (DNs), (B) high grade DNs, (C) DNs with hepatocellular carcinoma (HCC) foci in the right side, and (D) HCCs. Notice that clearly increased sinusoidal capillarization with the progression of human multistep hepatocarcinogenesis (LSAB, original magnification 100×).

2. Telomere length in human multistep hepatocarcinogenesis

The telomere terminal restriction fragment (TRF) length analysis demonstrated a gradual shortening of the telomere according the progression of human multistep hepatocarcinogenesis.

The telomere lengths of each lesion are summarized in Table 4 and Figure 4, with a representative result shown in Figure 5. Normal liver tissues showed TRF lengths ranging from 7.3 to 9.2 kb, which showed no significant correlation with patient ages, which ranged from 42 to 73 years. The TRF lengths ranged from 6.7 to 9.4 in CH, and from 4.6 to 10.5 in cirrhosis, which showed no significant differences from normal liver tissue. The cases of cirrhosis with co-existent HCCs showed a relatively shorter TRF lengths than those without co-existent HCCs (patients 2, 5 and 6). The TRF lengths in the LRNs ranged from 4.2 to 9.2 kb, which were similar to those of the cirrhosis.

Table 4. Summary of the terminal restriction fragment length and telomerase activity in human multistep hepatocarcinogenesis

	TRF length (kb)		Telomerase activity (A_{450}-A_{690})	
	range	Mean±SD	range	Mean±SD
Normal (*n* = 4)	7.3-9.2	8.3±0.92	0.5-0.7	0.6±0.08
CH (*n* = 3)	6.7-9.4	7.8±1.44	0.4-0.9	0.7±0.30
Cirrhosis (*n* = 10)	4.6-10.5	7.3±1.69	0.3-1.8	1.0±0.50
LRN (*n* = 7)	4.2-9.2	7.2±1.88	0.4-1.6	0.8±0.40
LGDN (*n* = 30)	3.6-10.4	7.2±1.97	0.2-2.9	0.9±0.56
HGDN (*n* = 6)	2.9-5.1	4.0±0.89	0.8-2.8	1.7±0.75
DN with HCC (*n* = 3)	3.6-5.3	4.5±0.85	0.8-3.1	2.3±1.39
HCC (*n* = 11)	3.4-9.3	5.5±1.89	0.6-3.3	2.1±0.97

Abbreviations: TRF, terminal restriction fragment; CH, chronic hepatitis; LRN, large regenerative nodule; LGDN, low grade dysplastic nodule; HGDN; high grade dysplastic nodule; DN, dysplastic nodule; HCC, hepatocellular carcinoma.

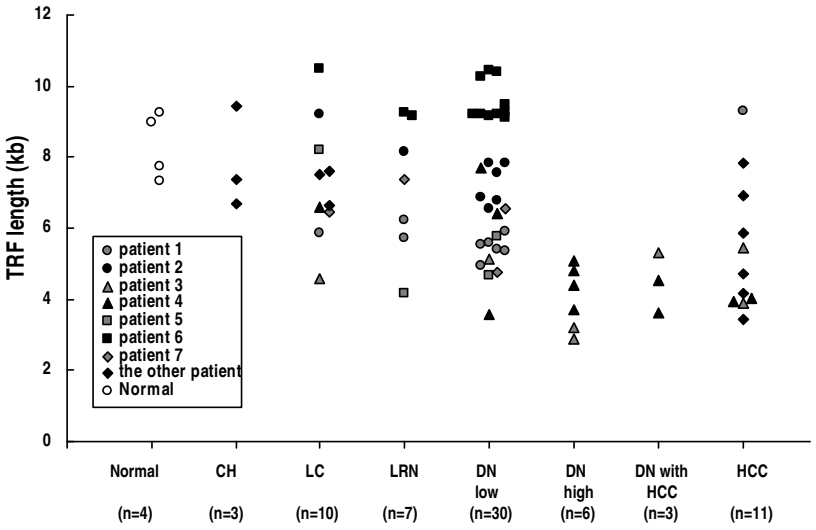

Figure 4. Comparison of the telomere length (in kilobases) in a normal liver, chronic hepatitis (CH), liver cirrhosis (LC), large regenerative nodules (LRNs), low grade dysplastic nodules (DNs), high grade DNs, DNs with hepatocellular carcinoma (HCC) foci and HCCs. The lesions are denoted by different symbols according to the patient number.

The low grade DNs showed a somewhat wider range of TRF lengths with most showing similar TRF lengths to those of LRNs and cirrhosis. Five (17%) low grade DNs (patients 1, 3, 4, 5 and 7) showed short TRF lengths of 3.6–5.1 kb, which overlapped with those of the high grade DNs, and they, except patient 5, had synchronous HCCs. There was no histological difference between the low grade DNs with a short TRF length compared to the others without. Four low grade DNs (patients 1 and 3) showed increased neoangiogenesis of grade 3 + sinusoidal capillarization, or 16–25 unpaired arteries per 10 fields at 100 x magnification, and had relatively short TRF lengths of 5.1, 5.3, 5.5 and 5.9 kb, although there was no statistically significant difference. The high grade DNs showed TRF lengths ranging from 2.9 to 5.1, and a significant shortening of the TRF length occurred in the transition of low grade DNs to high grade DNs (P = 0:0003). The TRF lengths in DNs with the HCC foci ranged from 3.6 to 5.3 and from 3.4 to 9.3 in HCCs.

There were no significant differences in TRF lengths of the high grade DNs, DNs with HCC foci and HCCs. No differences in TRF lengths were shown in relation the differentiation of HCCs.

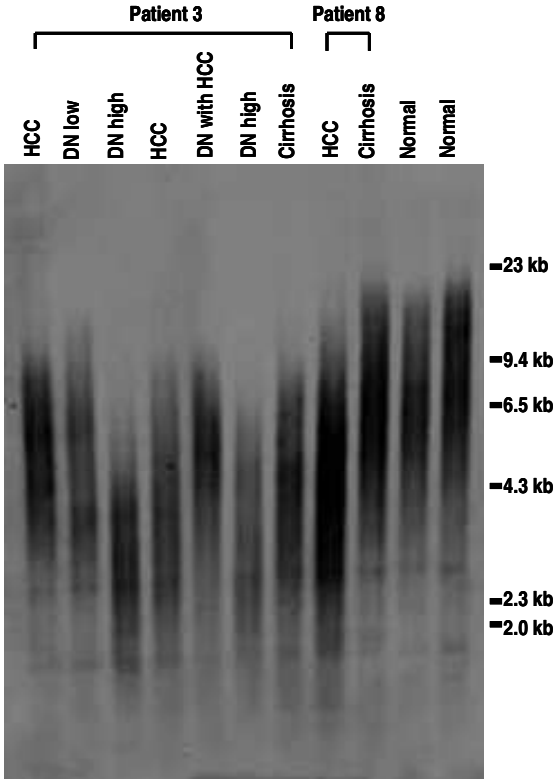

Figure 5. Southern blot analysis of the telomere length in dysplastic nodules (DNs) and hepatocellular carcinomas (HCCs). The telomere length was determined from each lesion by measuring the terminal restriction fragment (TRF) length. Genomic DNA (2 mg), digested with Hinf I, was hybridized with a digoxigenin-labeled and a telomerespecific probe $d(TTAGGG)_4$. The TRF lengths were determined using the IMAGE GAUGE software (Fujifilm, Tokyo, Japan), as described in materials and methods. The size markers are indicated on the right. The results from multiple synchronous nodules of DNs and HCC with cirrhosis in patient 3, HCC and cirrhosis in patient 8, and two normal liver tissues, are presented.

3. Telomerase activity in human multistep hepatocarcinogenesis

The telomerase activities (TAs) of each lesion are summarized in both Table 4 and Figure 6, and a representative result is shown in Figure 7. The normal livers displayed the range of TA from 0.5 to 0.7, which was similar to those of heat inactivated lysates or buffer only reactions.

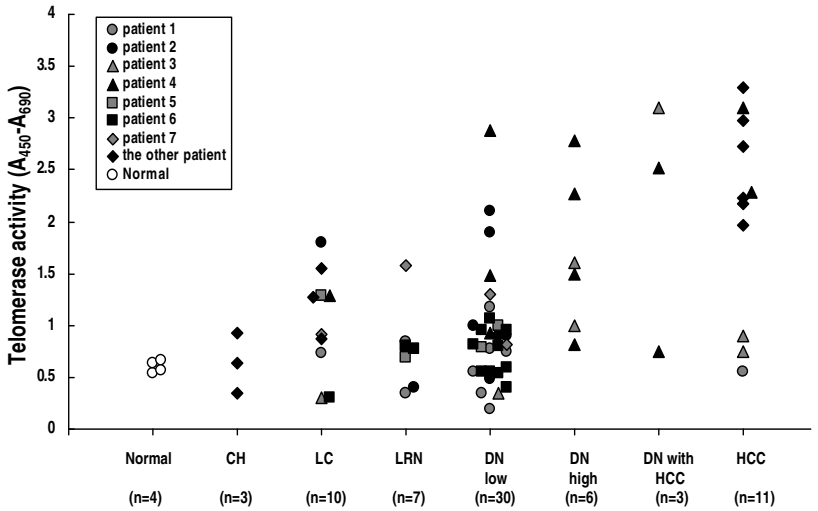

Figure 6. Comparison of the telomerase activity in a normal liver, chronic hepatitis (CH), liver cirrhosis (LC), large regenerative nodules (LRNs), low grade dysplastic nodules (DNs), high grade DNs, DNs with hepatocellular carcinoma (HCC) foci and HCCs. The lesions are denoted by different symbols according to the patient number.

Therefore, normal livers are considered to have no activity of telomerase. The TA ranged from 0.4 to 0.9 in CH, and from 0.3 to 1.8 in cirrhosis. The LRNs had similar levels of TA to those of cirrhosis, ranging from 0.4 to 1.6, with all lesions showing TA levels <2.0. The low grade DNs showed a somewhat wider range of TA levels, ranging from 0.2 to 2.9. Most of the low grade DNs showed similar TA levels to those of CH, cirrhosis, and LRNs.

However, two (7%) of them showed high TA level >2.0, where there was no or very sparse infiltration of inflammatory cells. There were no significant differences in the histology and neoangiogenesis between the low grade DNs with and without an increased TA. The high grade DNs showed TAs in the range from 0.8 to 2.8 and there was a significant increase of the levels of TA in the transition from low grade DNs to high grade DNs (P = 0:0086). The DNs with HCC showed TAs in the range from 0.8 to 3.1. The TA levels in the HCCs ranged from 0.6 to 3.3 and there were no significant differences relating to the differentiation of HCCs. The TA levels of high grade DNs showed no significant differences from those of DNs with HCC foci and HCCs, although there were two (33%) of high grade DNs, two (67%) of the DNs with HCC foci and seven (64%) of HCCs with TA levels over 2.0. No significant correlation was found when the TA levels were compared with the TRF lengths. The characteristic

telomere length pattern of alternative lengthening of telomeres, which results in homogeneity of the telomere length [14], was not found in three HCCs with low levels of TA from 0.6 to 0.9.

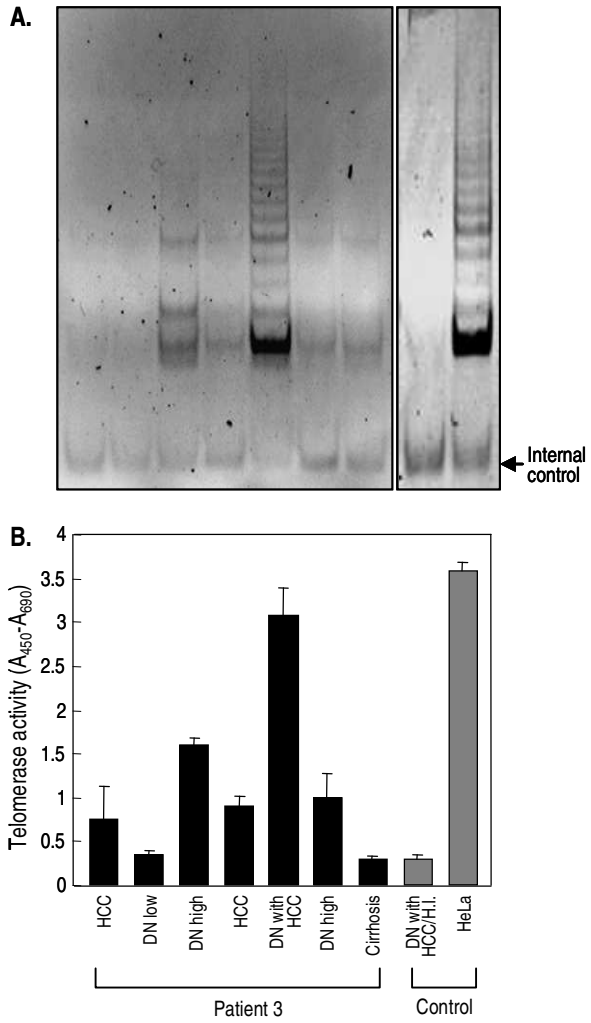

Figure 7. Telomerase activity in the multiple synchronous dysplastic nodules (DNs) and hepatocellular carcinomas (HCCs). The telomerase activity was determined using a TRAPeze Elisa telomerase detection kit, with a tissue extract (0.2 mg) from multiple synchronous nodules of DNs and HCCs and cirrhosis in patient 3. (A) The product underwent electrophoresis on an 12% polyacrylamide gel. The internal control is

indicated by the arrow head. (B) The telomerase activity in the tissue extracts shown in A was semi-quantified using Elisa according to the manufacturer's instructions. The reaction was repeated three to four times, and the resulting mean values and standard deviations are shown by the black bars. An equivalent amount of a HeLa cell extract was used as a positive control, and the tissue extract of the DN with HCC foci, heat-inactivated at 85 ℃ for 15 min prior to the TRAP assay, was used as negative control. The controls are indicated by the gray bars.

Ⅳ. Discussion

A gradual shortening of the TRF length, and an increase in the TA level, was found with the progression of human multistep hepatocarcinogenesis, and this was also evident in the multiple synchronous nodules of hepatocarcinogenesis of the same patient. Telomere shortening, and telomerase reactivation, occurs in the DNs during the early stages of hepatocarcinogenesis, and a significant change in the TA and TRF was founded in the transition from the low grade DNs to the high grade DNs. Significant increase in neoangiogenesis was also found in high grade DNs than in low grade DNs [7, 8]. High grade DNs and DNs with HCC foci showed similar high levels of TA and shortening of telomeres, which increased to the levels of the HCCs. The characteristics of high grade DNs are considered to be closer to those of the HCCs and different from those of the low grade DNs. Telomere shortening and telomerase activation are suggested to facilitate the diagnosis of precancerous/cancerous lesion on needle biopsy in a near future, in which only small amount of liver tissue is available. Studies on various aspects of low grade DNs, with respect to their proliferation, p-ras expression, ploidy, endothelial cell and extra cellular matrix alterations, have reported no similarities between them and HCCs, which is in contrast with the close similarities

found between high grade DNs and HCCs [6]. This suggests that low grade DNs might be related to regenerative nodules, and are less clearly implicated in hepatocarcinogenesis.

However, there was a significant statistical association between low grade DNs with co-existing HCCs, even though the high grade DNs were excluded from the analysis [15]. Hytiroglou et al. [11] reported that TA was positive or strong positive in 86% of LRNs/low grade DNs and weak positive in 62% of cirrhosis, in which low grade DNs were not separated from LRNs. Whereas Kitamoto and Ide [12] reported that TA was negative in all cases of chronic liver disease and positive in 30% of precancerous hepatic nodules, which include both low and high grade DNs.

In this study, low grade DNs, which were evaluated as a separate entity, showed somewhat wider ranges of TRF lengths and TA levels, and these overlapped with those of the CH, cirrhosis and LRNs in most cases. However, 17% of low grade DNs had shortened telomere lengths up to the levels of the high grade DNs, with 7% showing TA levels over 2.0. Hepatocellular nodules, with the telomeres shortened to the critical length, might suffer genetic changes due to the chromosomal instability [16, 17].

These genetic changes would make most cells senescent, but allow a small number of cells to undergo additional changes, such as telomerase reactivation, which facilitates the clonal development of immortal hepatocytes [18, 19] and the rate of

apoptosis was reported to be relatively lower in the low grade DNs than the surrounding cirrhotic nodules [20]. Some low grade DNs, at least those with shortened telomere lengths or increased TA levels up to those of high grade DNs, are considered to have a precancerous nature, even though the associated histological changes have still to occur, and close follow-up is required to clarify the clinical significance of these low grade DNs. However, the possibility that microscopic focus of high grade dysplasia was present in the remaining fresh tissue of low grade DNs can not be completely ruled out, because only one frozen section of each nodule with fresh tissue was examined before extraction in this study.

Cases of low grade DNs and cirrhosis with co-existing HCCs had relatively shorter telomere lengths compared to those without and it is considered to be a field effect of hepatocarcinogenesis. Considering that a telomere reduction reflects the replicative history in somatic cells [21], this result is in accordance with the report that the rate of HCC was high in cirrhotic patients with a high hepatocyte proliferative activity [22]. About 30% of cirrhotic nodules are monoclonal and early genomic aberrations occur in cirrhosis [18, 19, 23], therefore cirrhotic nodules with shortened TRF lengths might be more vulnerable to further genetic changes. Further study will be required to evaluate the biological characteristics of these nodules with shortened TRF length.

Ⅴ. Conclusion

This study demonstrates the telomere shortening and telomerase reactivation occur particularly in the course of transition from chronic liver disease and LGDNs to HGDNs, but not from HGDNs to HCCs. Accordingly the changes of telomerase length and TA might occur in the early stage of hepatocarcinogenesis.

Reference

1. E.H. Blackburn, The molecular structure of centromeres and telomeres. Annu Rev Biochem 1984;53:163–94.

2. K. Dubrana, S. Perrod and S.M. Gasser, Turning telomeres off and on. Curr Opin Cell Biol 2001;13;281–9.

3. J. Lingner, J.P. Cooper and T.R. Cech, Telomerase and DNA end replication: no longer a lagging strand problem? Science 1995;269:1533–4.

4. C.B. Harlery, A.B. Futcher and C.W. Greider, Telomeres shorten during ageing of human fibroblasts. Nature 1990;345:458–60.

5. International Working Party, Terminology of nodular hepatocellular lesions. Hepatology 1995;22:983–93.

6. N.D. Theise, Y.N. Park and M. Kojiro, Dysplastic nodules and hepatocarcinogenesis.

In: J.H. Lefkowitch, Editor, Clinics in liver disease. Liver histopathology 2002;1st ed.:497–512.

7. Y.N. Park, C.P. Yang, G.J. Fernandez, O. Cubukcu, S.N. Thung and N.D. Theise, Neoangiogenesis and sinusoidal ‘capillarization’ in dysplastic nodules of the liver. Am J Surg Pathol 1998;22:656–62.

8. M. Roncalli, E. Roz, G. Coggi, M.G. Di Rocco, P. Bossi, E. Minola et al., The vascular profile of regenerative and dysplastic nodules of the cirrhotic liver: implications for diagnosis and classification. Hepatology 1999;30:1174–8.

9. N. Miura, I. Horikawa, A. Nishimoto, H. Ohmura, H. Ito, S. Hirohashi et al., Progressive telomere shortening and telomerase reactivation during hepatocellular carcinogenesis. Cancer Genet Cytogenet 1997;93:56–62.

10. H. Kojima, O. Yokosuka, F. Imazeki, H. Saisho and M. Omata, Telomerase activity and telomere length in hepatocellular carcinoma and chronic liver disease. Gastroenterology 1997;112:493–500.

11. P. Hytiroglou, V. Kotoula, S.N. Thung, M. Tsokos, M.I. Fiel and C.S. Papadimitriou, Telomerase activity in precancerous hepatic nodules. Cancer 1998; 82:1831–8.

12. M. Kitamoto and T. Ide, Telomerase activity in precancerous hepatic nodules. Cancer 1999;85 :245–8.

13. P.A. Kruk, N.J. Rampino and V.A. Bohr, DNA damage and repair in telomeres: relation to aging. Proc Natl Acad Sci USA 1995;92:258–62.

14. T.M. Bryan, A. Englezou, L. Dalla-Pozza, M.A. Dunham and R.R. Reddel, Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat Med 1997;3:1271–4.

15. P. Hytiroglou, N.D. Theise, M. Schwartz, E. Mor, C. Miller and S.N. Thung, Macroregenerative nodules in a series of adult cirrhotic liver explants: Issues of classification and nomenclature. Hepatology 1995;21:703–8.

16. C.B. Harley, Telomere loss: mitotic clock or genetic time bomb?. Mutat Res 1991;256:271–82.

17. W.E. Wright and J.W. Shay, Telomere positional effects and the regulation of cellular senescence. Trends Genet 1992;8:193–7.

18. V. Paradis, I. Laurendeau, M. Vidaud and P. Bedossa, Clonal analysis of macronodules in cirrhosis. Hepatology 1998;28:953–8.

19. T. Ochiai, Y. Urata, T. Yamano, H. Yamagishi and T. Ashihara, Clonal expansion in evolution of chronic hepatitis to hepatocellular carcinoma as seen at an X-chromosome locus. Hepatology 2000;31: 615–21.

20. Y.N. Park, K.J. Chae, Y.B. Kim, C. Park and N. Theise, Apoptosis and proliferation in hepatocarcinogenesis related to cirrhosis. Cancer 2001;92:2733–8.

21. O. Isokawa, T. Suda, Y. Aoyagi, H. Kawai, T. Yokota, T. Takahashi et al., Reduction of telomeric repeats as a possible predictor for development of

hepatocellular carcinoma: convenient evaluation by slot-blot analysis. Hepatology 1999;30:408–12.

22. M.F. Donato, E. Arosio, E. Del Ninno, G. Ronchi, P. Lampertico, A. Morabito et al., High rates of hepatocellular carcinoma in cirrhotic patients with high liver cell proliferative activity. Hepatology 2001;34:523–8.

23. S.S. Thorgeirsson and J.W. Grisham, Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 2001;31:339–46.

ABSTRACT (in Korean)

인간의 다단계 간암발생과정 단계중 이형성 결절에서의 텔로미어 길이와 텔로머레이즈 활성도 조절기전

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연구목적 : 인간의 다단계 간암발생과정에 대한 연구가 최근 활발히 진행되면서 이형성 결절 (dysplastic nodule)이 간암의 전암병변 이라는 증거가 축적되고 있다. 그리고 텔로미어 길이감소와 텔로머레이즈 재활성화는 초기 간암발생과정의 초기에 나타나는 중요한 현상이지만 아직 그 기작은 정확히 알려져 있지 않다. 이러한 이유로 본 연구는 발암 신호로서의 텔로머레이즈 재활성에 의한 텔로미어 길이유지에 관해서 각 병변의 특징을 간암발생기전 에서 연구하였다.

연구재료 및 방법 : 텔로미어 길이 측정과 텔로머레이즈 활성도는 southern blot과 TRAPeze Elisa 텔로머레이즈 측정 키트를 사용하여 측정

하였다.

결과 : 텔로미어 길이와 텔로머레이즈 활성도는 고등도 결절과 저등도 결절 사이에서 의미 있게 다르다는 것이 확인되었다. 대부분의 저등도 결절은 만성간염, 경변증 그리고 거대재생결절의 텔로미어 길이와 텔로머레이즈 활성도가 비슷한 수준을 유지하였다. 하지만 저등도 결절의 17% 가 고등도보다 텔로미어 길이가 더 짧아졌으며 저등도결절의 7%만이 높은 텔러머레이즈 활성도가 나타났다. 고등도 결절에서의 텔로미어 길이와 텔로머레이즈 활성도 수준은 간세포암종 병소와 간세포암종에서 유래된 결절의 수준과 다르지 않았다.

결론 : 텔로미어 길이감소와 텔로머레이즈 재활성도 는 만성간염과 저등도 결절에서 고등도 결절로 변화될 때 부분적으로 일어난다. 하지만 고등도 결절에서 간세포 암종으로 발달할때는 그렇지 않다. 그러므로 텔로미어 길이감소와 텔로머레이즈 재활성도의 변화는 간암발생기전의 초기 단계 에서 일어날 가능성이 있다.

핵심 단어 : 텔로미어, 텔로머레이즈, 이형성 결절, 간세포암종, 거대재생결절, 간암발생기전, 경변증