

Aberration of X chromosome in liver neoplasm detected by fluorescence in situ hybridization

Jun Liu, Zhan-Min Wang, Shu-Fang Zhen, Xiao-Peng Wu, Dao-Xin Ma, Zhao-Hui Li, Bo Liu, Zhi-Lun Zhao and Yang Ke

Jinan, China

BACKGROUND: A diverse range of cytogenetic alterations of autosomal chromosomes has been reported to date. However, few studies have addressed the abnormalities of X chromosome in hepatocellular carcinoma (HCC) except sporadic reports on the deletion of band F1 in X chromosome, and the clonal analysis of methylation pattern of the X chromosome-linked human androgen receptor gene. Identification of specific X chromosome alterations during the course of neoplastic development would be essential to defining the genetic basis of HCC. Therefore, we studied the regularity of aberration of X chromosome in liver cancer.

METHODS: Hepatocarcinoma cellular lines and tumor tissues were detected respectively through DNA probes of X chromosome after fluorescence in situ hybridization (FISH).

RESULTS: Increased copies of X chromosome were observed in all samples, and four signals of hybridization were of the major type.

CONCLUSIONS: Increased copy number of X chromosome frequently occur in liver cancer. The relationship between copy number of X chromosome and liver cancer genesis needs further investigation. This study is the first of its kind determining the copy number of X chromosome in liver cancer by using FISH.

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KEY WORDS: liver neoplasm; X chromosome; fluorescence in situ hybridization

Author Affiliations: Department of General Surgery, Qilu Hospital, Shandong University, Jinan 250012, China (Liu J, Wang ZM, Wu XP, Ma DX, Li ZH, Liu B and Zhao ZL); Clinical Tumor Institute of Beijing University, Beijing 100014, China (Zhen SF and Ke Y)

Corresponding Author: Jun Liu, MD, PhD, Department of General Surgery, Qilu Hospital, Shandong University, Jinan 250012, China (Tel: 86-531-6921941 ext 5426; Fax: 86-531-6927544; Email: dr_liujun@hotmail.com)

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Introduction

Hepatocellular carcinoma (HCC) has a high incidence and poor prognosis in China.^[1-4] Elucidation of the basic genetic changes of human HCC is important for the understanding and treatment of this cancer. A diverse range of cytogenetic alterations of autosomal chromosomes has been reported to date. However, few studies have addressed the abnormalities of X chromosome in HCC except sporadic reports on the deletion of band F1 in X chromosome,^[5] and the clonal analysis of the methylation pattern of the X chromosome-linked human androgen receptor gene.^[6-16] Previously, we established a novel HCC cell line,^[17] which provided a useful model for study on liver cancer at the levels of cell and molecular biology. To study the mutation regulation of sex chromosome in liver cancer, we applied fluorescence technique in situ hybridization (FISH). After analysis of the nucleus type of chromosome, we found its number and construction appeared to be varied obviously. These results were found in cell lines and also confirmed by fresh parenchymal tumor tissues. A high frequency of X chromosome gains was observed in hepatoblastoma. Identification of specific X chromosome alterations during the course of neoplastic development would be essential to defining the genetic basis of HCC. Therefore, it is our purpose in this study to detect the aberration of X chromosome in liver neoplasm by FISH.

Methods

Hepatocarcinoma cellular lines and tumor tissues

Hepatocarcinoma cellular lines HCC-9903, EGHC-9901 and HCC-9810 were established at our laboratory of Qilu Hospital, Shandong University, China. Fresh parenchymal specimens of liver cancer were obtained from patients operated on at Qilu Hospital, Tumor Hospital of Shandong Province, Shandong Provincial Hospital, and Jinan Central Hospital, China. These speci-

mens were pathologically confirmed as HCC.^[18,19]

Preparation of chromosome

The fresh tumor tissues were frozen, sliced, and then kept in air for 5 minutes. The slices were put into the methyl alcohol/ice acetic acid (3:1) solution for 20-minute fixation. The solution was replaced by fresh one for another 20-minute fixation. Subsequently the slices were dried and kept at -20°C for use.

Labeling of probe

The labeling kit from Vysis Inc., USA was used for direct labeling of the probe according to the manufacturer's instructions. The orange-DUTP labeled probe was stored at -20°C for use.

FISH analysis

For precipitation of the probe, the X chromosome centromere probe ($5\ \mu\text{l}$), salmon sperm DNA ($1\ \mu\text{l}$), human placenta DNA ($2\ \mu\text{l}$), 3M sodium acetate ($1.2\ \mu\text{l}$), double distilled water ($4\ \mu\text{l}$) and ethanol ($30\ \mu\text{l}$) were mixed and cooled on ice for 15 minutes, then centrifuged at 12 000 rpm for 30 minutes. The supernatant was collected, dried with vacuum-pump, and then added with $3\ \mu\text{l}$ of sterilization ex-ion water, and $7\ \mu\text{l}$ of hybridizing solution VII. The mixture was denatured at 72°C for 5 minutes and cooled on ice for use. For hybridization, the slices were dehydrated in 70%, 80% and 100% ethanol for 5 minutes, respectively. The samples were dried and denatured at 72°C in 70% methylamine/ $2\times$ sodium chloride/sodium citrate (SSC) buffer (pH=

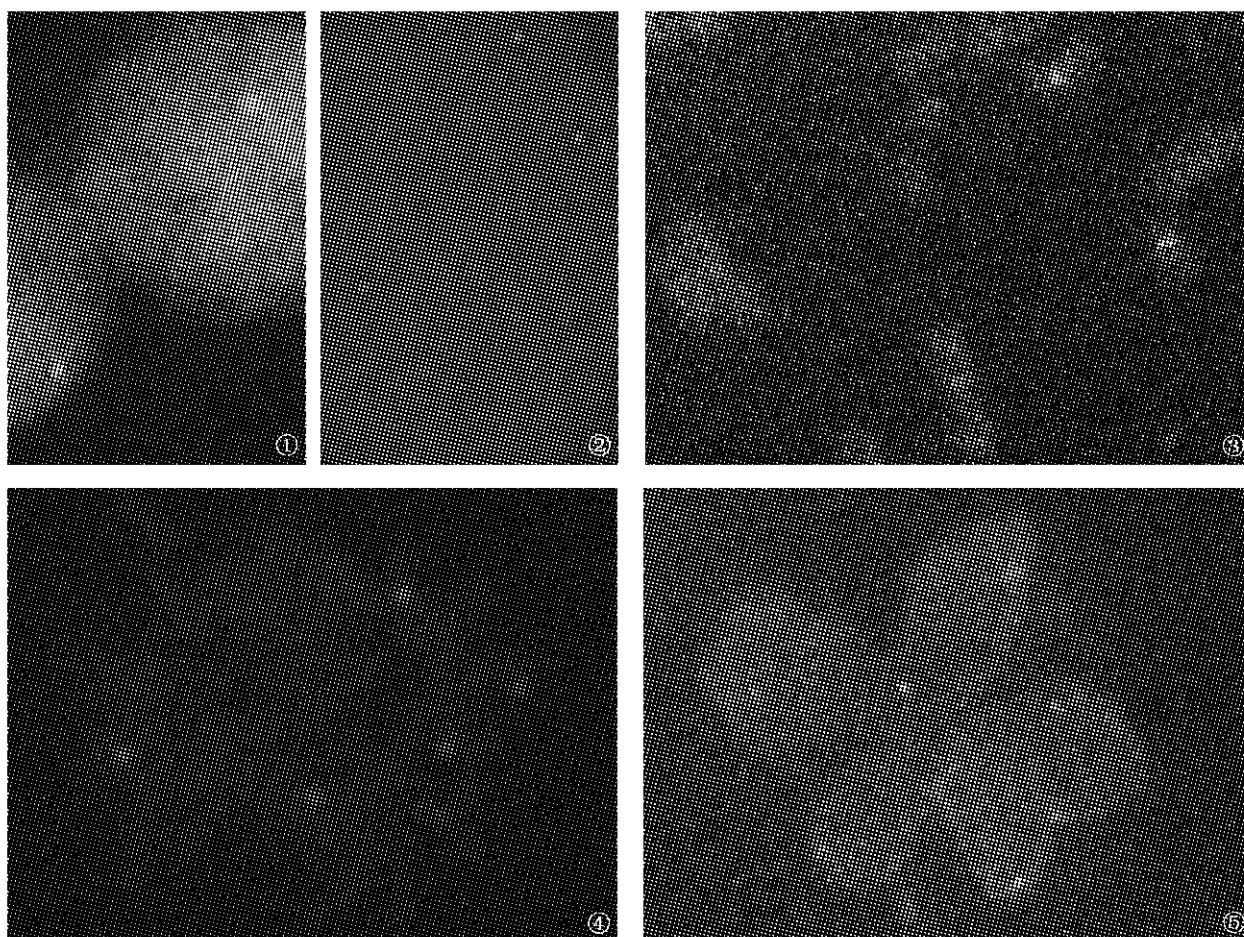


Fig. 1. The copies of X chromosome of normal lymphocyte under a fluorescence microscope (only one dot). One red bright dot represents one copy of X chromosome.

Fig. 2. The copies of X chromosome of HCC cell line under a fluorescence microscope (4 dots). One red bright dot represents one copy of X chromosome.

Fig. 3. The copies of X chromosome of HCC cell line under a fluorescence microscope (2 dots). One red bright dot represents one copy of X chromosome.

Fig. 4. The copies of X chromosome of HCC cell line under a fluorescence microscope (5 dots). One red bright dot represents one copy of X chromosome.

Fig. 5. The copies of X chromosome of HCC tumor samples under a fluorescence microscope. One red bright dot represents one copy of X chromosome.

7) for 3 minutes, then they were dehydrated by 70%, 80%, 100% ethanol. The denatured probe was put onto the slices, and kept at 37 °C in a wet-box overnight. The slices were washed at 72 °C with 0.4×SSC buffer/0.3% NP40 (pH=7) for 2 minutes, then with 2×SSC buffer/0.1% NP40 for 2 minutes and dried in air. The slices were restained with 10 µl of DAPI (0.05 µg/ml). 15 minutes–2 hours later, cells were counted microscopically. Hundred interphase nuclei of tumor cells were counted and normal lymphocytes were used as controls.

Principles of counting

The signal of each nucleus was recorded at 6 different levels according to its pieces. The following criteria were followed: counting of non-overlap nucleus; independent signal; non-counting of injured nucleus or plasm inside nucleus; consistent signal strength for calculation; counting of paired leaf signal as one; counting of average in four quadrants so as to decline the error caused by hybridization and examination to the lowest limit.

Results

Cell nucleus and chromosome were stained in light blue under a fluorescence microscope. Red bright dots were shown in the nuclei of positive cells. The dot indicated the position of X chromosomes. The copies of X chromosomes of control lymphocytes were found to be normal (Fig. 1). The probes were found stable. The results of hepatocarcinoma cellular lines are presented in Table 1 and Figs. 2–4. The results of 12 tumor samples are shown in Table 2 and Fig. 5. The results indicated that the increased copies of X chromosome were common in hepatocarcinoma cellular lines, with 4 copies in most cases. The same results were obtained using tumor samples.

Table 1. FISH analysis of X chromosome copies of hepatocarcinoma cellular lines

Sample	Copies of X chromosome (entries)						Amplification X chromosome (%)
	0	1	2	3	4	≥5	
HCC-9903	0	1	1	7	81	10	99
EGHC-9901	0	0	9	60	21	10	100
HCC-9810	1	0	0	8	70	21	99

Discussion

Sex hormone and its receptor, especially the inducible male hormone receptor, have been widely reported to be related to liver cancer. But the importance of the abnormalities of X chromosome has not yet been investigated in liver cancer. For a long time, sex chromosome has been considered to decide sex, and few studies have paid attention to the gene in sex chromosome, which is related to human growth and development. Recently, the gene of male hormone receptor, the gene of CD40 gamete, the γ-gene of IL-2 receptor were found to locate in X chromosome. Therefore, the studies on abnormalities of X chromosome in liver cancer are helpful to identify the causes of liver cancer and mechanism of the male hormone at gene level.

The method of zoster show of chromosome is an important technique for chromosome analysis. But it is challenged in the analysis of tiny or complicated chromosome abnormalities and the settlement of source of small marked chromosomes. Tumor cell is not easy to enter the split-phase during culture and get the analysis phase as well. It may cause the failure of analysis because of the poor appearance or unclear band of chromosome. Recently, FISH as an accurate and efficient technique has been used for the study of complicated chromosome mutation. Hybridization in situ and fluorescence techniques

Table 2. FISH analysis of X chromosome copies of tumor samples

Case	Sex	Age (γ)	Operation date	Copies of X chromosome (entries)						Amplification X chromosome (%)
				0	1	2	3	4	≥5	
Wang	M	44	2001-06-21	0	14	13	14	45	14	86
Li	M	60	2001-07-03	0	18	8	26	34	14	82
Li	M	47	2001-07-20	0	19	14	18	30	19	81
Zhang	M	55	2001-07-24	0	24	12	14	40	10	76
Guo	M	62	2001-07-26	0	17	13	25	36	9	83
Ding	M	63	2001-07-30	0	6	16	35	22	21	94
Wang	F	47	2001-08-01	0	3	13	19	35	30	84
Liu	M	45	2001-08-01	0	24	13	26	27	10	76
Han	M	55	2001-08-02	0	16	12	9	46	17	84
Liu	M	48	2001-08-04	0	18	7	38	24	13	82
Yu	M	54	2001-08-06	0	26	12	26	26	10	74
Wang	M	62	2001-08-06	0	17	9	20	36	18	83

are used in FISH, which could replace isotope hybridization in situ owing to its high sensitivity and strong signal. FISH has been widely used to study the changes in count and structure of split phase cells, chromosome quantity, and gene changes of interphase cells. The application of FISH in the study of liver cancer in the recent years^[20-26] has revealed mutation of 1, 2, 3, 4, 6, 7, 8, 9, 16, 17, 18 and 20 chromosomes. To the present, however, no report has been published on copies of X chromosome in liver cancer. The use of FISH to study the X chromosome of hepatocarcinoma cellular lines in this study demonstrated that the number of copies was significantly increased, with 4 copies in most cases, indicating that X chromosome may play a role in the occurrence and development of liver cancer. This finding is consistent with that reported by Ochiai^[12] and Paradis.^[27] They found that the mutation of male hormone receptor on the X chromosome in patients with chronic hepatitis was related to liver cancer. We further examined fresh parenchymal tumor and obtained the same result. Thus, the error margin that may be caused by the biological characteristics of cells was excluded. This finding indicates that sex hormone and involved genes need further investigation.

Competing interest

The author or authors do not choose to respond to the statements listed in Instructions for Authors.

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