

Dual fluorescence in situ hybridization in detection of HER-2 oncogene amplification in primary hepatocellular carcinoma

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BACKGROUND: Molecular cytogenetics of oncogene HER-2 amplification in primary hepatocellular carcinoma (HCC) is still unknown. The aim of this study was to investigate the frequency of HER-2 oncogene amplification in primary HCC and its relations to clinicopathological parameters and prognosis.

METHODS: Forty-two surgical samples from patients with primary HCC were detected for their HER-2 oncogene amplification. The number of chromosome 17 and their ratio were tested by dual fluorescence in situ hybridization (FISH) technique, and then the correlations between HER-2 amplification, clinicopathological characteristics and prognosis were analyzed statistically.

RESULTS: HER-2 oncogene amplification was detected in 9 (21.4%) of the 42 primary HCCs, including 4 patients with high copy (HC) (9.5%) and 5 patients with low copy (LC) (11.9%). HER-2 amplification was associated significantly with tumor size and postoperative survival time of HCC patients ($P < 0.05$), and the presence of HER-2 gene amplification was correlated with postoperative relapse ($P = 0.257$), but not related to sex, age, AFP level, HBV infection, histopathological grading and clinical staging of HCC patients ($P > 0.05$). The HER-2 oncogene copy was examined in 31 (73.8%) of the 42 primary HCCs, consisting of 9 patients with HER-2 amplification (21.4%) and 22 patients with aneuploidy (52.4%). No significant relations were observed between the HER-2 oncogene copy, patient sex, tumor size, histopathological grading, clinical staging, postoperative relapse and survival time ($P > 0.05$); but the HER-2 oncogene copy was correlated significantly to age, AFP level and HBV infection ($P < 0.05$).

CONCLUSIONS: There are a lower frequency of HER-2

oncogene amplification and a higher frequency of chromosome 17 aneuploidy in primary HCC. HER-2 oncogene amplification may be involved in the development and progression of large HCC in some patients, and seems to be a valuably independent prognostic factor predicting the recurrence and poor survival in patients with large HCC.

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KEY WORDS: hepatocellular carcinoma, primary; HER-2 oncogene; amplification; dual fluorescence in situ hybridization

Introduction

Amplification is one of the major forms of oncogene activation, which together with tumor suppressor gene inactivation determines the molecular genetics of tumor occurrence and development.^[1-4] In general, oncogenes are localized at the amplified regions of chromosomes whereas tumor suppressor genes (TSGs) at the deleted zones of chromosomes in some tumors.^[5] Many oncogenes and TSGs such as ras gene, Rb gene, BRCA1 gene and so on were cloned successfully from the amplified or deleted regions of chromosomes in many kinds of cancer, respectively. The oncogene HER-2 located at chromosome 17q11.2-q12 as an oncogene homologous to epidermal growth factor receptor gene (EGFR) codes for a cell membrane glycoprotein kinase that promotes the ability of tumor cell infiltration and migration.^[6] HER-2 amplification and over-expression of its oncoprotein are revealed in mammary carcinoma, ovarian carcinoma and other tumors as non-small cell lung cancer and are reported to be closely related to the prognosis.^[7,8]

Though immunohistochemical analysis^[9] and comparative genome hybridization (CGH)^[10] have been applied to trace the over-expression of HER-2 and amplification of chromosome 17q in hepatocellular carcinoma (HCC), no genetic evidence of HER-2 amplification in HCC has been found using fluorescence in situ hybridization (FISH) technique worldwide. In recent years, such proto-oncogenes as c-fos and c-myc were found to over-express in cirrhotic tissues.^[11] In this stu-

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dy, HER-2 amplification was quantitatively detected with dual color FISH in 42 patients with primary HCC and its clinical significance was also discussed.

Methods

HCC specimens

The 42 patients (38 men and 4 women) were subjected to hepatectomy, and specimens were collected from May 1996 to December 1996. Twenty-five patients were over 45 years old and 17 below 45 years (average 47 years). Pathologically confirmed, the patients were classified into phase I (6 patients), II (33) or III (2) according to the international TNM staging. In 30 patients, the diameter of tumors was more than 5 cm. In the remaining 12 patients, the size of tumors was less than 5 cm. The level of serum alpha-fetoprotein (AFP) was higher than 25 $\mu\text{g/L}$ in 29 patients and lower than 25 $\mu\text{g/L}$ in 13 patients, respectively. HBsAg was positive in 28 patients and negative in 13. HCC was classified pathologically into grade I (1 patient), II (11), and III (13). Follow-up for 3 and half a year showed that 14 patients survived more than 2 years and 28 died in 2 years.

Single cell suspensions and preparation of FISH glass slides

A modest amount of HCC specimens was harvested and physically smashed into single cell suspension at low temperature in a RPMI1640 medium. The cell suspension was centrifugated before being collected and subsequently treated under a low osmolarity and fixed with 3:1 methanol/acetyl acid. The single cell suspension was dropped on the pre-treated glass slides and air-dried for 3 to 7 days before use.

Preparation of dual color FISH probes

The Path Vysion™ HER-2 DNA Probe Kit consisted of a 190 kb red fluorescence labeled HER-2 gene located at chromosome 17D11. 2-q12 and a silk-wear granule probe CEP17 at chromosome 17. The probe was purchased from the Vysis Company, USA and was authorized by the USA FDA to be applied clinically for quantitative detection of HER-2 amplification.

Dual color FISH detection

The examination was performed according to the method established by our laboratory^[12] and the manufacturer's instructions to the use of the probe kit. The detailed description about pre-treatment of glass slides, probe denaturalization, pre-hybridization, sealing of hybridized slices, re-staining of washed slices and microscopic observation of polychrome was reported previously.^[13-15] The meta-imaging software of 3.6 edition was

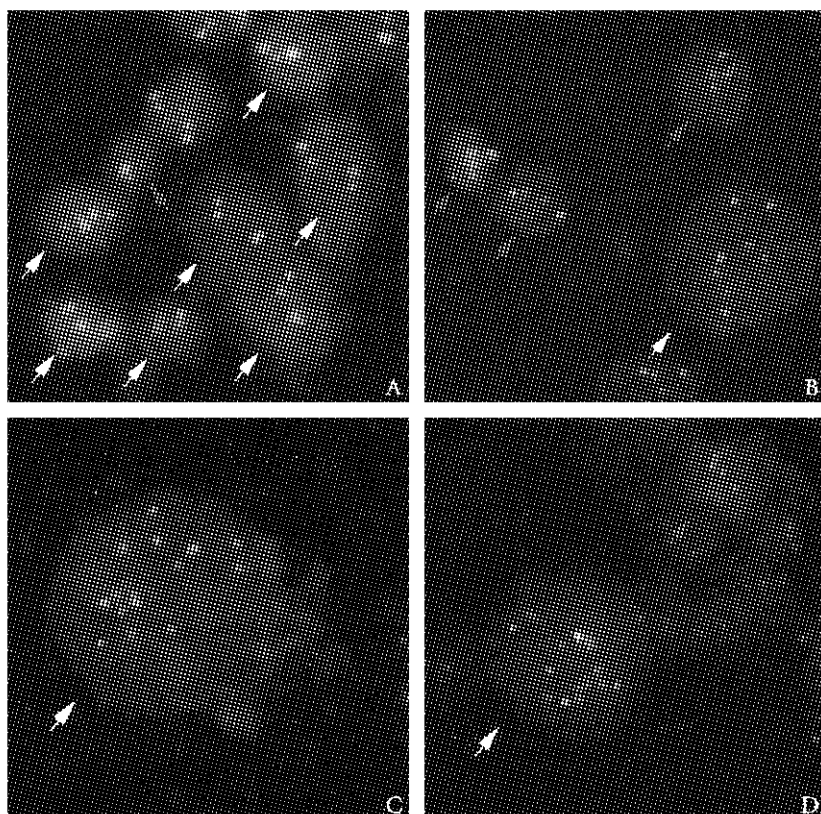


Fig. Profile of HER-2 oncogene amplification in primary HCC detected by dual FISH. **A**: interphase FISH pattern of diploid HCC nuclei with two copies of HER-2 allele. White arrows showing cancerous nuclei with two red fluorescent hybridization signals for HER-2 and two green fluorescent hybridization signals for CEP-17, while blue arrow indicating non-cancerous lymphocyte. **B**: interphase FISH pattern of aneuploid HCC nuclei with HER-2 copy gains, and multiple red hybridization dots for HER-2 and green dots for CEP17 ($\text{HER-2/CEP17}=1.0$). **C**: interphase FISH pattern of HCC nuclei with low copy amplification of HER-2 oncogene, and multiple red FISH dots for HER-2 oncogene and a few of green FISH dots for CEP17 ($1.6 < \text{HER-2/CEP17} < 2.0$). **D**: interphase FISH pattern of HCC nuclei with high copy amplification of HER-2 oncogene, and many red hybridization dots for HER-2 and less green dots for CEP17 ($\text{HER-2/CEP17} > 2.0$).

used for acquiring, saving and analyzing signal pictures of fluorescence hybridization.

Quantitative criteria for HER-2 amplification

In each case, a total of 100 integrated, dispersed nuclei without superposition were observed. HER-2 amplification was defined when more than 20% of the nuclei displayed abnormal hybridization signals. The ratio of red-labeled HER-2 hybridization signal number to green-labeled CEP 17 signal number was used as a quantitative index for HER-2 amplification. Greater HER-2/CEP 17 ratio implied stronger HER-2 amplification. The cut-off value of positive amplification was set at 1.6. HER-2 amplification was defined when the HER-2/CEP17 ratio was above 1.6. A ratio between 1.6 and 2.0 means a low copy amplification and that of more than 2.0 indicates a high copy amplification. A ratio of 1.0 together with separately only two hybridization signals detectable for both HER-2 and CEP17 suggests nor-

mal disomy 17. When the ratio was 1.0 but multi-hybridization fluorescence signals were detected for both HER-2 and CEP17, aneusomy 17 or polysomy 17 was defined as gained HER-2 copies.

Statistical analysis

Pearson's χ^2 test or Fisher's exact test was performed using the 8.0 edition SPSS statistical software for the diversity prominence comparison of frequency variation on numeric data and correlation analysis of crossing-data. The difference was statistically significant when *P* value was less than 0.05.

Results

HER-2 amplification in HCC detected by dual color FISH

Both red (HER-2) and green (CEP17) fluorescence

Table 1. Detection of pattern and ratio of two-colour signals for HER-2 oncogene and chromosome 17 centromere by interphase dual FISH

Case	Number of signals					Chromosome 17 (%)					HER-2/CEP17	Ratio	Type
	HER-2 (%)					Chromosome 17 (%)							
	2	3	4	5	≥6	2	3	4	5	≥6			
T11	2	10	75	8	5	8	8	68	10	6	4/4	1.0	Tetrasomy 17
T12	6	3	5	82	4	3	7	10	73	7	5/5	1.0	Pentasomy 17
T13	25	20	35	12	8	28	18	40	10	4	3-4/3-4	1.0	Aneusomy 17
T14	10	45	25	8	12	52	10	12	15	11	3-4/2	1.7	LC*
T15	8	22	42	15	13	30	48	10	8	4	3-4/2-3	1.8	LC
T16	15	40	23	12	10	10	50	20	15	5	3-4/3-4	1.0	Aneusomy 17
T17	5	60	15	13	7	2	68	10	12	8	3/3	1.0	Trisomy 17
T18	12	18	35	30	5	8	15	38	27	12	4-5/4-5	1.0	Aneusomy 17
T20	10	65	10	10	5	8	70	9	10	3	3/3	1.0	Trisomy 17
T25	8	72	12	8	0	2	68	15	7	8	3/3	1.0	Trisomy 17
T28	18	12	28	34	8	15	30	29	16	10	4-5/3-4	1.8	LC
T29	7	10	15	30	38	40	28	12	15	5	5-8/2-3	2.6	HC**
T30	35	25	30	5	5	30	28	32	10	0	3-4/3-4	1.0	Aneusomy 17
T31	25	27	23	15	10	24	29	19	18	10	3-4/2-3	1.9	LC
T33	22	7	31	12	28	25	5	28	10	33	4-8/4-8	1.0	Aneusomy 17
T34	34	10	26	30	0	35	7	25	32	1	4-5/4-5	1.0	Aneusomy 17
T35	8	10	51	18	13	5	7	60	14	14	4/4	1.0	Tetrasomy 17
T36	15	12	19	28	26	18	16	15	29	22	5-6/5-6	1.0	Aneusomy 17
T37	13	10	22	38	17	16	10	26	40	8	4-5/4-5	1.0	Aneusomy 17
T38	16	61	7	12	4	12	66	5	15	2	3/3	1.0	Trisomy 17
T39	11	10	19	27	33	35	30	15	5	15	5-12/2-3	3.2	HC
T42	12	8	17	39	24	11	27	33	20	9	5-12/3-5	2.8	HC
T47	15	5	59	14	7	13	10	62	11	4	4/4	1.0	Tetrasomy 17
T49	7	31	42	10	10	6	34	40	15	5	3-4/3-4	1.0	Aneusomy 17
T51	12	38	27	12	11	8	41	25	11	15	3-4/3-4	1.0	Aneusomy 17
T53	20	9	17	33	21	24	31	21	17	7	5-10/2-4	3.0	HC
T55	18	41	22	10	9	15	45	21	8	11	3-4/3-4	1.0	Aneusomy 17
T57	13	77	10	0	0	10	80	7	2	1	3/3	1.0	Trisomy 17
T60	18	10	22	48	12	13	8	21	50	8	4-5/4-5	1.0	Aneusomy 17
T61	10	30	26	27	7	6	29	21	30	14	3-5/3-5	1.0	Aneusomy 17
T62	2	10	38	28	22	8	62	12	8	10	4-6/3	1.7	LC

*: LC means low copy amplification; **: HC means high copy amplification.

hybridization signals were observed in an absolute majority of the nuclei tested. According to the quantitative criteria for HER-2 amplification and the number or ratio of fluorescence signals (red or green), the results were classified into normal disomy HCC without HER-2 amplification (Fig. A), aneusomy HCC with gained HER-2 copies (Fig. B), HCC with low copy HER-2 amplifi-

cation (Fig. C) and HCC with high copy HER-2 amplification (Fig. D). A few of normal disomy lymphocyte nuclei were also observed in part of the specimens (Fig. A-D). The detailed results from the 31 patients with abnormal dual color signals were seen (Table 1).

HER-2 amplification and its correlation with clinical

Table 2. The levels of HER-2 oncogene amplification in 9 HCC patients and their clinicopathological characteristics

Case	Ratio *	Amplification	Sex	Age	Staging	Tumor size	AFP level	HAA status $\Delta\Delta$	Histopathology	Relapse	Survival time
T14	1.7	LC**	Male	58	I	5×5×5	68	-	HCC (II)	17 months	1.5 years
T15	1.8	LC	Male	57	II	8×5×5	1197	-	HCC (III)	No	5 months
T28	1.8	LC	Male	52	II	6.5×6×5	10	+	HCC (II-III)	12 months	12 months
T29	2.6	HC Δ	Male	53	II	6.5×6.5×6	5820	+	HCC	4 months	1.5 years
T31	1.9	LC	Male	61	II	10×8×6	10	+	HCC	4 months	1.5 years
T39	3.2	HC	Male	49	II	10×8×8	331	+	HCC	D $\#$	40 days
T42	2.8	HC	Male	44	II	6×5×5	-	+	HCC (III)	1 months	4 months
T53	3.0	HC	Male	38	II	10×7×5	19532	+	HCC (II)	D	14 days
T62	1.7	LC	Male	47	II	15×13×8	1131	+	HCC (I)	5 months	8 months

* (HER-2/CEP17); HER-2/CEP17 ratio; ** (LC): low copy amplification; Δ (HC): high copy amplification; $\Delta\Delta$ (HAA); HBsAg; # (D): death shortly after operation.

Table 3. Correlations of HER-2 gene amplification and gains in HCC to its clinicopathological parameters and prognosis

Clinicopathological parameters	n	HER-2 amplification		P value	HER-2 gain		P value
		Positive cases	Percentage		Positive cases	Percentage	
Sex							
Male	38	9	23.7	0.272	28	73.7	0.955
Female	4	0	0.0		3	75.0	
Age (years)							
>45	25	7	28.0	0.208	15	60.0	0.014 *
<45	17	2	11.8		16	94.1	
AFP ($\mu\text{g/L}$)							
>25	29	6	20.7	0.892	26	89.7	<0.01 *
<25	13	3	23.1		5	38.5	
HAA							
+	28	7	25.0	0.489	24	85.7	0.023 *
-	13	2	15.4		6	46.1	
Tumor size (cm)							
>5	31	9	29.1	0.044 *	24	77.8	0.695
<5	11	0	0.0		7	70.0	
Histopathology							
HCC (grade I)	1	1	100.0	0.153	1	100.0	0.824
HCC (grade II)	11	3	27.3		8	72.7	
HCC (grade III)	13	2	15.4		10	76.9	
Clinical staging							
Stage I	6	1	16.7	0.683	4	66.7	0.674
Stage II	33	8	24.2		25	75.8	
Stage III	2	0	0.0		1	50.0	
Postoperative recurrence							
Yes	27	6	22.2	0.257	20	74.1	0.618
No	13	1	7.7		9	69.2	
Survival time (years)							
<2	28	9	32.1	0.046 *	20	71.4	0.620
>2	14	0	0.0		11	78.6	

*: $P < 0.05$.

pathological features and prognosis of HCC

In the 42 patients with hepatocellular carcinoma, in 9 patients, HER-2 amplification (21.4%) was detected by dual color FISH including high copy amplification in 4 patients (9.5%) and low copy amplification in 5 (11.9%). Their HER-2 amplification and clinical pathological features showed huge HCC with a diameter of over 5 cm. A total of 8 patients were in clinical stage II except 1 in stage I (Table 2). In the 4 patients with high copy HER-2 amplification, two (T39 and T53) died shortly after operation, and the remaining two (T29 and T42) had recurrence of HCC in half a year. It is worth noting that none of the 9 patients with HER-2 amplification survived more than 2 years post operation. Gained HER-2 copies were revealed in 31 (73.8%) of the 42 patients, including 9 patients with HER-2 amplification (21.4%) and 22 patients with chromosome 17 aneusomy or polysomy (52.4%). HER-2 amplification and copies gaining as well as their correlation with clinical pathological features and prognosis of HCC were analysed (Table 3). Our data suggested that HER-2 amplification is not related to sex, age, AFP level, HBV infection, postoperative recurrence or clinical staging ($P > 0.05$), but to the 2-year survival rate after operation. The patients with HER-2 amplification had a lower 2-year survival rate (0/9) than did those without HER-2 amplification (42.4%), $P = 0.046$. Compared with those without HER-2 amplification, the patients with HER-2 amplification tended to have a large size of tumor ($P = 0.085$). In addition, a gained copy of HER-2 showed no relations to sex, clinical staging, tumor size, postoperative recurrence or survival time, but to age, AFP level and HBV infection ($P < 0.05$).

Discussion

As one of the major manners of oncogene activation, oncogene amplification forms the molecular genetic basis of oncoprotein over-expression. HER-2, an oncogene located at chromosome 17q11.2-q12, has been found by many researchers to be amplified in various kinds of tumors such as mammary carcinoma, ovarian carcinoma and lung cancer. It is thought to be closely related to metastasis, recurrence and prognosis of part of these tumors.^[16-20] In mammary carcinoma, HER-2 amplification has become an independent prognostic factor for some patients receiving different doses of chemotherapy after operation. It has been an important evidence for postoperative supplementary chemotherapy and immunotherapy.^[21] Dual color FISH technique was used in this study to verify HER-2 amplification in HCC at molecular genetic level, and HER-2 amplification was found to be correlated with the prognosis of HCC patients.

HER-2 amplification existed in 21.4% (9/42) of

HCC patients in this study. This rate is close to that (19%) in patients with Barrett esophagus related adenoma but a little bit lower than in those with mammary carcinoma, ovarian carcinoma and lung cancer (20%–40%). It is slightly higher than in patients with cervical carcinoma and melanoma (5%–20%). Research into HER-2 and HCC is currently limited in serology, immunohistochemistry, and CGH. Molina et al.^[22] reported abnormal concentrations of HER-2 protein antigen in 26.7% of HCC patients. Immunohistochemical analysis revealed HER-2 over-expression as high as 92.3% in HCC by Tang et al.,^[9] but no obvious relationship was observed between HER-2 expression and HCC recurrence or migration. As for CGH results, chromosome 17q amplification was as high as 30% in HCC,^[10] but no more evidence could verify HER-2 gene amplification at 17q11.2-q12. With fluorescence labeled HER-2 genome DNA probes, we observed in this study HER-2 gene (17q11.2-q12) amplification at a single nucleus level. This provided molecular genetic substantiation for the results from serology, immunohistochemistry and CGH. In HCC, HER-2 amplification displayed no correlation with such clinical features as AFP level or HBV infection ($P > 0.05$) but tended to appear in huge HCC ($P = 0.085$) and was responsible to the short survival period in these patients. The 2-year survival rate (0/9) was lower in the patients with HER-2 amplification than those without HER-2 amplification (42.4%), $P = 0.046$. Possibly, AFP concentration and HBV infection did not influence HER-2 amplification, but HER-2 amplification might be a potential independent prognostic factor that could influence the outcome of HCC patients. Heinze et al.^[23] determined HER-2 protein level in advanced HCC patients with ELISA method. The 1- and 2-year survival rates were 20% and 10% in high protein level patients but 56% and 22% in low protein level patients, separately ($P < 0.05$). Thus HER-2 protein expression was considered a valuable predictor for prognosis. In this study we also observed increased “copy gain”^[24,25] because of aneusomy 17/polysomy 17 in 52.4% of HCCs; but this presented no obvious correlation with the 2-year survival rate after operation ($P = 0.901$). Perhaps the numeric abnormality of chromosome does not essentially reflect the outcome of the patient, whereas structural changes in chromosome such as gene amplification would fundamentally influence the outcome. It is shown that “copy gain” is not an ideal prognostic factor and cannot be used to predict the short-term survival of postoperative patients. “Copy gain” and its correlation with AFP level or HBV infection ($P < 0.05$) as well as whether HBV infection under the circumstance of high AFP level could result in aneusomy 17 needs further study.^[26] Zekri et al.^[27] suggested that HCV-1a and HCV-4III could induce high level expression of HER-2 and ultimately result in HCC

occurrence.

The limitation of immunohistochemistry determines the instability in repeated examination and thus could not essentially reflect the variation of genetic material.^[28-30] As a new molecular genetic technique, dual color FISH could not only make it possible to illustrate the nucleus structure in the precondition of preserving the nucleus shape, but also allow quantitative evaluation of target gene amplification using the numeric probe of silk-wear granule on the gene containing chromosome. A direct method is provided to observe the number and distribution of mono-nucleus genes and the clone variation of chromosome structure or amount of different tumor cells in the same tissue. Some researchers suggested that the two methods should be combined in evaluation of gene amplification.^[31]

HCC is one of the most malignant neoplasm of all tumors and shows a trend toward recurrence and metastasis after resection of HCC, and so the postoperative patients with HCC have a poorer prognosis.^[32,33] With the rapid advance of molecular biology, molecular diagnosis, gene therapy and molecular prevention are becoming increasingly part of our patient management and will eventually complement or replace in part the existing diagnostic, therapeutic and preventive strategies.^[34]

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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