DNA topoisomerase IIα and Ki67 are prognostic factors in patients with hepatocellular carcinoma

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Abstract. The present study was designed to determine the significance of DNA topoisomerase IIα (TopoIIα) and Ki67 in hepatocellular carcinoma cells (HCCs). The present study included 353 patients with HCC. The association of clinicopathological data with the expression of TopoIIα and Ki67 by immunohistochemistry was analyzed by χ2 test. Cox multivariate proportional hazards regression analysis and Kaplan-Meier analysis were performed with all the variables to derive risk estimates associated with overall survival (OS)/recurrence-free survival (RFS) and to control for confounders. TopoIIα and Ki67 were detected in the nuclei of the tumor cells. With TopoIIα, 35.7% of cells exhibited high expression, which was associated with tumor-node-metastasis stage, tumor size and α-fetoprotein level. With Ki67, 37.1% of cells exhibited high expression, which was associated with tumor-node-metastasis stage, tumor size and α-fetoprotein level. Correlation was identified between the expression level of TopoIIα and Ki67 in HCCs (r=0.444). Multivariate analysis revealed that high TopoIIα expression is a prognostic indicator for RFS [hazard ratio (HR), 2.002; 95% confidence interval (CI), 1.429-2.806] and OS (HR, 2.749; 95% CI, 1.919-3.939), and high Ki67 expression is a prognostic indicator for OS (HR, 1.816; 95% CI, 1.273-2.589). The TopoIIα-low group had a significantly increased RFS rate (55.6 vs. 31.7%) and OS rate (66.5 vs. 23.8%) compared with the TopoIIα-high group. The OS rate was increased in the Ki67-low group compared with the Ki67-high group (67.0 vs. 26.5%). Expression of TopoIIα and Ki67 are independent prognostic factors for survival in HCCs. TopoIIα was positively associated with Ki67 expression.

Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-associated mortality globally, and it is more prevalent in men compared with women (1). According to one estimation, 745,500 mortalities occurred among the 782,500 new liver tumor cases worldwide during 2012 (2). Currently, surgical resection is the optimal treatment strategy to offer long-term survival outcomes for patients with HCC. However, the clinical behavior of HCC may vary. In certain patients, the disease runs an aggressive course with a survival of several months, whereas other patients may have a relatively slow clinical evolution and survive for >5-10 years following diagnosis. An improved understanding of the pathogenesis and identification of novel biomarkers for HCC may provide physicians treating this disease with additional therapeutic options. Although it has been proposed that several molecular markers, including α-fetoprotein (AFP), may be associated with the prognosis of HCC, their clinical applications are limited (3-5). Therefore, previous studies have focused on seeking new biomarkers for the prognosis and therapeutic targeting of HCC (6-8).

The proliferation status of tumor cells is an important parameter that reflects the biological characteristics of the tumor, and affects the prognosis and efficiency of treatment of the tumor directly. DNA topoisomerases are enzymes essential for DNA replication, transcription and recombination, as well as for chromosome compaction and segregation (9). Several families and subfamilies of the two types (I and II) of DNA topoisomerases have been described (9). There are two isoforms of topoisomerase II in humans, α and β, coded on chromosomes 17q21-22 and 3p24, respectively (10,11). TopoIIα is essential for the survival of actively growing cells. Enzyme concentrations are upregulated markedly during periods of cell proliferation, and TopoIIα levels increase over the cell cycle and have an increased peak in the G2/M phase (9). Considering the previously mentioned role of TopoIIα in cell division and its increased expression in the G2/M phase of the cell cycle, immunohistochemical staining for TopoIIα may be useful in the determination of cell proliferation, and eventually malignant transformation (9-11). Previous studies have considered TopoIIα as a prognostic factor in patients with esophageal squamous cell carcinomas, breast cancer, clear cell renal cell carcinoma and nasopharyngeal carcinoma (12-15).
One of the established proliferation markers is Ki67 protein, which may be detected within the cell nucleus. As Ki67 protein is present during all active phases of the cell cycle (G1, S, G2 and mitosis) but is absent in the resting cell (G0), it is an effective marker for determining the growth fraction of a given cell population (16). In a multi-ethnic sample of patients with breast cancer, Ki67 was revealed to be a significant independent prognostic marker (17).

Various studies have been performed with TopoIIα or Ki67 individually to investigate their role as prognostic markers for the survival of patients with HCC. However, thus far, whether TopoIIα or Ki67 affect the long-term survival of patients with HCC is controversial. The present study investigated TopoIIα and Ki67 expression and evaluated their utility in predicting the outcomes of patients with HCC.

Materials and methods

Patients and tumor samples. HCC tumor samples (353 in total) were obtained from patients who underwent hepatic surgical resection without preoperative chemotherapy at the Fuzhou General Hospital (Fujian, China) between February 2003 and October 2012. Using the International Union Against Cancer tumor-node-metastasis (TNM) classification system (18) to classify resected specimens, 251 cases at stage I/II and 102 cases at stage IIIa were identified. Histological grades were classified as well/moderately-differentiated (n=329) and poorly-differentiated (n=24). All the patients enrolled in the present study conformed to the following criteria: No tumor thrombus in the portal vein; only one tumor lesion; 0-1 Eastern Cooperative Oncology Group score prior to surgery (liver function grade Child-Pugh class A; no distant metastasis, ascites or hepatic encephalopathy); no cancer treatment prior to surgery, and pathology confirmed as primary HCC following surgery; complete clinical records and follow-up information available; no history of other tumors; radical resection. Radical resection was defined as follows: All lesions were resected and confirmed by intraoperative ultrasonography; pathology proved to be negative; the tumor thrombus following two branches were able to be resected. Exclusion criteria were: Insufficient liver tissue on the biopsy specimen for extra analysis or insufficient clinical data regarding patient outcome; and without history of hepatitis B virus infection.

The clinical data analysis staff members were blinded to the TopoIIα and Ki67 expression levels in the liver cancer samples, and the laboratory and analysis staff members did not view the clinical data. The present study protocol was approved by the Ethical Committees of Fuzhou General Hospital (Fuzhou, China) and informed consent was obtained from all patients.

Follow-up subsequent surgery. Patients who underwent hepatectomy between February 2003 and October 2012 were subjected to close clinical observation, including abdominal computed tomography imaging, AFP level and liver function tests at 2-4-month intervals. The follow-up end date was October 1st 2014, and the median follow-up time was 34 (range, 2-122) months. The follow-up was conducted via telephone or questionnaire as supplementary methods.

Immunohistochemical (IHC) analysis. IHC methods previously described by Schmilovitz-Weiss et al (19), for Ki67 assay were used to analyze the expression of Ki67 using anti-Ki67 antibody (Maixin Biotechnology Co., Ltd., Fujian, China; clone no., MID-1; dilution, 1:130) to replace the antibody. The same method was used to analyze the expression of DNA TopoIIα, using anti-DNA TopoIIα antibody (Maixin Biotechnology Co., Ltd; clone no., 5F6; dilution, 1:150) to replace the antibody.

Evaluation of IHC staining. The 353 stained tissue sections (4-µm thick) were evaluated on separate occasions by two pathologists with no previous knowledge of any patient information. Semi-quantitative IHC detection was used to determine TopoIIα protein level with a 4-point scale, as follows: No positive cells, 0; <25% positive cells, 1; 25-50% positive cells, 2; ≥50% positive cells, 3. HCC tissue samples graded 0 or 1 were judged as low TopoIIα expression, whereas those graded 2 or 3 were regarded as high TopoIIα expression.

As Ki67 expression was mostly homogenous, it was scored as a percentage of positively-stained cells, based on the following standards: (-), cancer cells unstained or stained <10% (cancer cells stained ≥10% were identified as positive); (+), cancer cells stained 10-25%; (++), cancer cells stained 26-50%; (+++), cancer cells stained 51-75%; (++++) cancer cells stained >75%. HCC tissue samples with (-) or (+) Ki67 expression levels were judged as low Ki67 expression; samples with (++), (+++) or (++++) Ki67 expression were regarded as having high Ki67 expression.

Statistical analysis. Analyses were conducted using SPSS statistical software (version 20.0; IBM SPSS, Armonk, NY, USA). The data are presented as the median and range. Categorical data were analyzed using a χ² test or Fisher's exact test. The correlation between TopoIIα and Ki67 expression was analyzed using Spearman's rank correlation test. Overall survival (OS) and recurrence-free survival (RFS) rates were evaluated by the Kaplan-Meier method, and the differences were examined with the log-rank test. Univariable risk ratios with 95% confidence intervals (CIs) were calculated using Cox proportional hazards regression models with enter-stepwise selection. To evaluate the prognostic value of TopoIIα and Ki67 expression, a Cox multivariate proportional hazards regression analysis was performed with all the variables adopted for their prognostic significance by univariate analysis with enter-stepwise selection. P<0.05 was considered to indicate a statistically significant difference.

Results

Demographic features and clinicopathological data. The present study cohort included 309 men and 44 women with a median age of 53 years (range, 13-81 years) and a median tumor size of 4 cm (range, 1-26 cm). Serum AFP level was ≤400 µg/l in 66.0% of the patients and >400 µg/l in 34.0% (normal ranges, <20.0 ng/ml) The tumors were well/moderately-differentiated in 93.2% of the patients and poorly-differentiated in 6.8%; 26.1% of the tumors were on the left side and 73.9% were on the right side; and tumor TNM stage was I/II in 71.1% of the patients and IIIa in 28.9%.
Table I. Association between TopoIIα/Ki67 expression and characteristics of patients (n=353).

| Characteristic                        | Ki67 expression | TopoIIα expression | P-value |
|---------------------------------------|-----------------|---------------------|---------|
|                                       | Low, n (%)      | High, n (%)         |         |
|                                       |                 |                     |         |
| Age                                   |                 |                     |         |
| ≤55 years                             | 126 (56.8)      | 80 (61.1)           | 0.427   |
| >55 years                             | 96 (43.2)       | 51 (38.9)           | 0.218   |
| Gender                                |                 |                     |         |
| Male                                  | 197 (88.7)      | 112 (85.5)          | 0.373   |
| Female                                | 25 (11.3)       | 19 (14.5)           | 0.921   |
| Histological grade                    |                 |                     |         |
| Well-/moderately-differentiated       | 210 (94.6)      | 119 (90.8)          | 0.176   |
| Poorly-differentiated                 | 12 (5.4)        | 12 (9.2)            | 0.489   |
| Serum AFP level (µg/l)                |                 |                     |         |
| ≤400                                  | 159 (71.6)      | 74 (56.5)           | 0.004   |
| >400                                  | 63 (28.4)       | 57 (43.5)           | 0.017   |
| Tumor location                        |                 |                     |         |
| Left                                  | 56 (25.2)       | 36 (27.5)           | 0.641   |
| Right                                 | 166 (74.8)      | 95 (72.5)           | 0.641   |
| TNM stage                             |                 |                     |         |
| I/II                                  | 168 (75.7)      | 83 (63.4)           | 0.014   |
| IIIa                                  | 54 (24.3)       | 48 (36.6)           | 0.009   |
| Survival                              |                 |                     |         |
| Deceased                              | 72 (32.4)       | 81 (61.8)           | <0.001  |
| Alive                                 | 150 (67.6)      | 50 (38.2)           | <0.001  |
| Tumor size                            |                 |                     |         |
| ≤5 cm                                 | 143 (64.4)      | 67 (51.1)           | 0.014   |
| >5 cm                                 | 79 (35.6)       | 64 (48.9)           | 0.001   |

AFP, α-fetoprotein; TNM, tumor-node-metastasis; TopoIIα, topoisomerase IIα. *The statistical test used to produce these P-values was χ² test.

Figure 1. Observation of nuclear Ki67 staining in a case of HCC. (A) High expression of Ki67 in HCC (magnification, x200). (B) High expression of Ki67 in HCC (magnification, x400). Observation of nuclear TopoIIα staining in a case of HCC. (C) High expression of TopoIIα in HCC (magnification, x200). (D) High expression of TopoIIα in HCC (magnification, x400). HCC, hepatocellular carcinoma; TopoIIα, topoisomerase IIα.
Ki67 expression and clinicopathological parameters of HCC are presented in Table I and Fig. 1. Ki67 expression was detected in the nuclei of the tumor cells in the HCC tissues (Fig. 1A and B). Among the 353 HCC tissues, 131 (37.1%) exhibited high expression and 222 (62.9%) had low expression levels. The results revealed that Ki67 expression was associated with TNM stage (P=0.014), tumor size (P=0.014) and high AFP level (P=0.004). However, no association was observed between Ki67 and age, sex, tumor location or histological grade.

TopoIIα expression was also detected in the nuclei of the tumor cells in the HCC tissues (Table I; Fig. 1C and D). Of the 353 HCC tissues, 126 tissues (35.7%) exhibited high expression and 227 (64.3%) showed low expression. Similar to the expression pattern of Ki67, TopoIIα was associated with TNM stage (P=0.009), tumor size (P=0.001) and AFP level (P=0.017), whereas no association was observed between TopoIIα and age, sex, tumor location or histological grade.

TopoIIα is associated with Ki67 expression in HCC. The IHC results revealed that TopoIIα expression rate was positively correlated with Ki67 (Spearman's correlation coefficient, r=0.444; P<0.001).

RFS. The results of the survival analyses are presented in Tables II and III and Fig. 2. In the present study, the 5-year RFS rate of the patients in the study cohort was 47.2%. During the observation period, 171 (48.4%) patients developed tumor recurrence. As predicted, a highly significant association was detected between tumor size (HR, 1.933; 95% CI, 1.429-2.613;
P<0.001), TNM stage (HR, 2.843; 95% CI, 2.088-3.871; P<0.001), TopoIIα (HR, 2.320; 95% CI, 1.708-3.151; P<0.001), Ki67 (HR, 1.760; 95% CI, 1.297-2.389; P<0.001) and the development of tumor recurrence by the univariate analysis method.

By multivariate analysis of all the factors that were significant in the univariate analysis, the independent prognostic indicators were TNM stage (HR, 3.757; 95% CI, 2.033-6.945; P<0.001) and TopoIIα (HR, 2.749; 95% CI, 1.919-3.939) and Ki67 (HR, 2.829; 95% CI, 2.093-3.995) were poor predictors for OS, while age, sex, histological grade, tumor location and serum AFP level had no prognostic significance for OS. Multivariate analyses of factors demonstrated that the TNM stage (HR, 3.757; 95% CI, 2.033-6.945), TopoIIα (HR, 2.749; 95% CI, 1.919-3.939), tumor size (HR, 1.938; 95% CI, 1.203-3.124) and Ki67 (HR, 1.816; 95% CI, 1.273-2.589) were the independent prognostic indicators. The TopoIIα-low group had a significantly increased OS rate compared with the TopoIIα-high group (66.5 vs. 23.8%; P<0.001). The OS rate was also significantly increased in the Ki67-low group compared with in the Ki67-high group (67.0 vs. 26.5%; P<0.001).

### Discussion

HCC is a common malignant tumor with a high mortality rate in humans (20,21). Due to the vast heterogeneity of patients

| Table III. 5-year overall survival percentage according to clinicopathological variables. |
|---------------------------------------------------------------|
| Characteristic | Cases, n (n=353) | 5-year OS, % | P-value |
|----------------|-------------------|-------------|---------|
| Age            |                   |             |         |
| ≤55 years      | 206               | 51.0        | 0.319   |
| >55 years      | 147               | 54.1        |         |
| Sex            |                   |             |         |
| Male           | 309               | 51.4        | 0.178   |
| Female         | 44                | 59.1        |         |
| TNM stage      |                   |             | <0.001  |
| I/II           | 251               | 61.7        |         |
| IIIa           | 102               | 29.3        |         |
| Tumor location |                   |             | 0.821   |
| Left           | 92                | 59.0        |         |
| Right          | 261               | 50.9        |         |
| Histological grade |           |             | 0.150   |
| Well/moderately differentiated | 329 | 51.5 |         |
| Poorly differentiated | 24  | 63.1 |         |
| Serum AFP level (µg/l) |       |             | 0.281   |
| ≤400           | 233               | 53.7        |         |
| >400           | 120               | 49.8        |         |
| Tumor size     |                   |             | <0.001  |
| ≤5 cm          | 210               | 65.7        |         |
| >5 cm          | 143               | 33.5        |         |
| TopoIIα expression |             |             | <0.001  |
| Low            | 227               | 66.5        |         |
| High           | 126               | 23.8        |         |
| Ki67 expression|                   |             | <0.001  |
| Low            | 222               | 67.0        |         |
| High           | 131               | 26.5        |         |
| TopoIIα-Ki67 expression |       |             | <0.001  |
| TopoIIα (low) or Ki67 (low) | 270 | 61.9 |         |
| TopoIIα (high) and Ki67 (high) | 83  | 18.2 |         |

P-value obtained using the Log-rank test. AFP, α-fetoprotein; TNM, tumor-node-metastasis; OS, overall survival; TopoIIα, topoisomerase IIα.
with HCC, prognosis following surgery may differ, and current clinicopathological factors, including AFP, TNM stage and tumor size, are not able to accurately predict the outcomes of all patients with HCC (22). Thus, there is an urgent requirement to find new biomarkers for prognosis and therapeutic HCC targets.

The proliferation status of tumor cells is an important parameter that reflects the biological characteristics of the tumor, affects prognosis and the efficiency of treating the tumor directly (16). TopoIIα and Ki67 are markers of cell proliferation in normal and neoplastic tissues; their expression levels may be used as a predictive parameter (9,16).

It is established that Ki67 protein is a traditional proliferation marker, and various studies have explored the predictive value of the Ki67-labeling index (17,23-27). The associations between Ki-67 and clinicopathological features, as well as its prognostic significance in numerous cancer types, including breast cancer, pituitary adenomas, laryngeal carcinoma, adrenocortical carcinoma, ovarian carcinoma and lymphoma, have been investigated, and the results were similar (17,23-27). These previous studies demonstrated that the expression of the Ki67 protein was associated with a more advanced tumor stage, underlining the importance of Ki-67 as a prognostic parameter.

A meta-analysis of 54 studies with a total of 4,996 patients examined the effect of high Ki67 on HCC clinicopathological features and patient DFS, RFS and OS (28). The results revealed that a high Ki67 level was significantly associated with advanced HCC stage, which included poor differentiation, large tumors and an increased number of tumor nodes, with metastasis, cirrhosis and vein invasion In accordance with previous studies, the present study demonstrated that Ki67 expression is associated with TNM stage, tumor size and AFP level. Using multivariate analysis, Ki-67 was an independent factor for OS, but was not associated with RFS. In addition, the OS rate was increased in the Ki67-low group compared with the Ki67-high group, which may indicate that a high expression level of Ki67 is associated with poor prognosis in patients with HCC.

Similar to Ki67, TopoIIα is another proliferation marker that is considered to function in growth-dependent processes, including DNA replication and chromosome segregation, due to its ability to break the DNA double helix. TopoIIα is involved in the DNA replication process and chromosome segregation, which are crucial for cell growth and development (11,9) and may facilitate tumor cell growth (29,30). Enzyme concentrations are upregulated dramatically during periods of cell proliferation, and they are associated with the proliferation of the cells (31,32). The prognostic value of TopoIIα has been pointed out by different researchers in various types of cancer (33).

While studying patients with HCC, Wong et al (34) identified that TopoIIα expression was associated with advanced histological grading, microvascular invasion and early age onset of the malignancy, and that high TopoIIα protein score (Grade 3) was associated with non-responsiveness to chemotherapy and early disease-associated mortalities. Similar to their study, the present study revealed that TopoIIα may be detected in the nuclei of tumor cells in HCC tissues. Of the 353 HCC tissues in the present study, 126 (35.7%) displayed high expression. In addition, TopoIIα was associated with TNM stage, tumor size and high AFP level. By multivariate analysis, TopoIIα remained an independent factor for OS and RFS. In addition, the low-TopoIIα group had increased OS and RFS rates compared with the high-TopoIIα group.

In 2001, Watanuki et al (35) reported an association between the TopoIIα index and the Ki-67 index in 70 resected HCC samples, and patients with lower TopoIIα had prolonged DFS and OS times. Most recently, the prognostic significance of TopoIIα in HCC was further elucidated by Panvichian et al (36), who studied gene aberrations of TopoIIα and protein expression levels of TopoIIα and Ki-67 in tumor and corresponding non-tumor tissues. The overexpression of
TopoIIα was only identified in tumor tissues; TopoIIβ gene amplification was not detected in tumor or non-tumor tissues, and overexpression of TopoIIα was associated with Ki-67. Based on previous studies, the present study investigated whether TopoIIα expression in HCC is associated with the expression of other, more established markers of proliferation (such as Ki67). Notably, the present study revealed that TopoIIα expression rate is positively associated with Ki67, and the presence of TopoIIα predicted reduced RFS, which is in accordance with earlier work by Watanuki et al (35).

The present study was limited by its retrospective nature, and all the patients in the present cohort were confined by the inclusion criteria. As the number of poorly differentiated tumors in the present study was small, additional research with a larger sample of HCC cases from different centers may be of great value.

In conclusion, TopoIIα and Ki67 may serve as an unfavorable prognostic factor for patients with HCC undergoing curative tumor resection. Examination of TopoIIα and Ki67 may help to stratify subgroups of patients and establish targeted therapies. Thus, undertaking adjuvant treatment early in Ki67 (high) or TopoIIα (high) patients following surgery may prolong survival. However, the underlying mechanism requires additional study.

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