RESEARCH ARTICLE

Tissue Microarray Immunohistochemical Profiles of p53 and pRB in Hepatocellular Carcinoma and Hepatoblastoma

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Abstract

The tumour suppressor genes, p53 and pRb, are known to play important roles in neoplastic transformation. While molecular routes to the uncontrolled growth of hepatocytes, leading to primary liver cancer have generated considerable interest, the roles of p53 and pRb mutations in hepatocellular carcinoma (HCC) and hepatoblastoma (HB) remain to be clarified. We examined the immunohistochemical expression of p53 and pRb gene products in 26 HCC and 9 HB, sampled into tissue microarray blocks. 10 (38%) of 26 HCC showed > 10% tumour nuclear staining for p53 protein, 3 of these also being HbsAg positive. Conversely, none of 9 HB expressed nuclear p53 immunopositivity. Some 24 (92%) HCC and 8 (89%) HB showed loss of pRb nuclear expression. Two of the 26 HCC and one of the 9 HB showed >10% tumour nuclear staining for pRb protein. Our results suggest that p53 does not have an important role in the development of HB but may contribute in HCC. There is also loss of pRb expression in the majority of HCC and HB, supporting loss of pRb gene function in the hepatocarcinogenesis pathway. However, a comparison of the staining profiles of p53 and pRb proteins in HCC and HB did not reveal a consistent pattern to differentiate between the two types of tumours immunohistochemically. Hence the use of p53 and pRB protein expression has no contribution in the situation where there is a diagnostic difficulty in deciding between HCC and HB.

Keywords: Liver cancer - hepatoblastoma - p53 - pRB - tumour suppressor genes - tissue microarray

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common human cancer globally particularly in Asia and Africa, while hepatoblastoma (HB) is the most common malignant childhood liver tumour, accounting for two-thirds of primary hepatic tumours in children (Litten and Tomlinson, 2008; Jemal et al., 2011). In the Malaysian setting, HCC ranks among the five most common adult cancers and accounts for >90% of all primary liver cancers, being particularly important because of its abysmal prognosis. HB ranks as a common childhood cancer after leukaemia, retinoblastoma, neuroblastoma and Wilm's tumour (Lim et al., 2008).

Histologically HCC can assume trabecular-sinusoidal, pseudoglandular-acinar or solid-scirrhous patterns, with hepatic, clear or pleomorphic cells. The tumours are usually graded as well-differentiated, moderately-differentiated, poorly-differentiated and undifferentiated types using the WHO classification (Ishak et al., 1994).

Malignant childhood tumours are histologically unique, as they tend to have an embryonal rather than pleomorphic-anaplastic appearance and frequently exhibit features of organogenesis specific to the tumour site. Hepatoblastoma may be epithelial (fetal, embryonal and fetal, macrotrabecular and anaplastic small-cell) or mixed epithelial- mesenchymal in type (Ishak and Glunz, 1967; Tanaka et al., 2013). These patterns frequently co-exist in the same tumour. The rare macrotrabecular type resembles adult hepatocellular carcinoma, but usually occurs in association with fetal or embryonal epithelial types. In the event of difficulty in differentiating between this subtype of HB and a childhood HCC, immunohistochemistry has not been helpful as both tumours express α-fetoprotein, cytokeratins and carcinoembryonic antigen (CEA).

While necroinflammatory agents such as hepatitis B and C, alcohol and aflatoxin B1 are known aetiological agents of HCC, such agents are not known to be important in the causation of HB (Litten and Tomlinson, 2008). It is mooted that HB, being an embryonal tumour, may occur as a result of germline mutations of tumour suppressor genes such as p53 or pRB (Brodeur, 1995; Terada et al., 2009). Even so, it is intriguing that malignant transformation of hepatocytes by viral and inflammatory agents should lead to a morphologically different cancer (HCC) from HB although the same cell of origin (hepatocyte) is involved.

Over the years, it has become clear that several profound molecular events occur in a multi-step fashion in the process of carcinogenesis. Although a variety of molecular genetic events have been described in

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experimental and human hepatocarcinogenesis, a consistent sequence of changes has not been established (Pan et al., 2011; Shiraha et al., 2013), although changes in p53 have been documented (Chittmittrapap et al., 2013; Thongbai et al., 2013). As both HCC and HB are important tumours in surgical pathology practice, we sought to investigate whether the immunohistochemical profile of p53 and pRB gene protein expressions in HCC and HB may cast light on their carcinogenesis and diagnostic utility.

Materials and Methods

This is a retrospective, immunohistochemical study of HCC and HB tumour material archived at the Department of Pathology, University Malaya Medical Centre (UMMC) utilizing tissue microarray (TMA) technology. The research proposal was reviewed and approved by the Medical Ethics Committee of the University of Malaya Medical Centre, as part of a larger project on pathological profiles of cancer.

Selection of tissue samples

Archived cases of histopathology-proven HCC and HB were identified and retrieved from the surgical pathology records of the Department of Pathology, University of Malaya over an eleven-year period. A total of 90 HCC and 26 HB cases were listed in the archives during the study period. The records and materials were reviewed to (1) avoid duplication of cases, (2) confirm the histological diagnosis, and (3) determine whether there was sufficient material for immunohistochemical studies.

Needle biopsies were excluded in order to preserve the integrity of the diagnostic archive. Furthermore, they were not suitable for TMA. The paraffin blocks with the largest amount of tumour content were selected for histological review. 81 cases were excluded from the study due to insufficiency of tissue for TMA study or non-retrievable tissue blocks. Finally, 26 cases of typical (unequivocal histology) HCC carcinoma and 9 HB were included in the study. One paraffin block of tumour material was selected from each case.

Demographic information (age, sex, ethnic group) was retrieved from the histopathology reports. The patients' identities were anonymised (no names recorded). Hepatitis B surface antigen status of the patients was obtained through the laboratory information system.

Controls

Control cases comprised (1) one normal liver tissue from an archived block from a five-month-old boy with biliary atresia (negative control for p53), (2) one archived block of colonic adenocarcinoma (positive control for the p53), (3) one normal tonsillar tissue from an archived block from a 24-year-old woman with chronic tonsillitis (positive control for pRb protein) and (4) one archived block of retinoblastoma (negative control for pRB).

Tissue microarray

All 35 selected test blocks and the 4 control blocks were processed in a manual tissue microarray processor (MTA-1 Manual Tissue Arrayer, Beecher Instruments Inc.) at the Department of Pathology, Singapore General Hospital. Prior to coring, all paraffin blocks of the selected cases were sectioned and stained with hematoxylin and eosin (H&E) and viewed for the presence of optimally representative tumour regions. For each case, two representative areas were selected. The areas of interest were marked out on the slides. The corresponding regions were circled on the donor block with a marker pen. 2 cores of intermediate size (1.0mm diameter) were taken, one from each circled area, for each case.

This resulted in the production of two microarray tissue blocks: 1 block containing the 26 HCC cases with 4 control cases, and the second other block containing the 9 HB cases with the same 4 control cases (Figure 1).

Immunohistochemistry for p53 and pRB

5-micrometer thick sections were cut from the microarray blocks and mounted on poly-L-lysine coated slides. They were stained with haematoxylin, eosin and for p53 protein and pRB protein expressions after microwave antigen retrieval (99°C with citrate buffer at pH 6.0 for 20 minutes), using standard immunoperoxidase methodology based on the Dako® Envision detection kit which utilized commercial monoclonal mouse anti human p53 (Dako, 1:50 dilution) and pRb (Dako, 1:50 dilution) antibodies.

Histological and immunohistochemical evaluation

The stained sections were reviewed microscopically by two investigators (AAH and LLM) to classify (subtype) the cancers according to the WHO classification for HCC (Ishak et al., 1994) and the classification system of Ishak and Glunz (1967) for HB. The percentage of cancer cells which show nuclear immunopositivity as well as the pattern, and intensity of protein expressions were recorded. Sections stained for the two parameters (p53 and pRb proteins) were assessed using a scoring system as follows: N/A= uninterpretable (e.g: loss of section); 0= 0% cells staining; 1= <10% of cells staining; 2= 10-25% of cells staining; 3= 26-50% of cells staining; 4= 51-75% of cells staining; 5= >75% of cells staining.

Score results for duplicate cores were consolidated into one score with positive staining always superceding a negative or uninterpretable result.

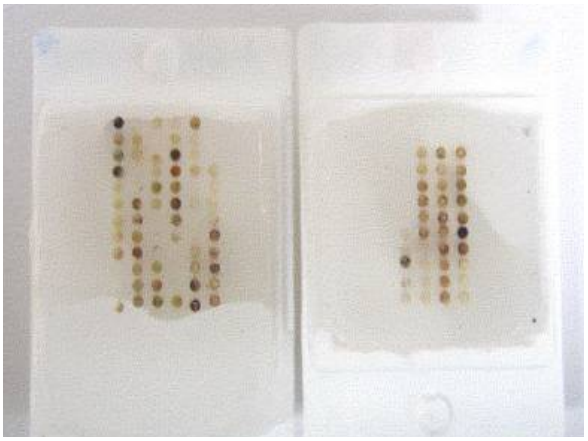

Figure 1. Microarray Tissue Blocks. Left: hepatocellular carcinoma and control cores; Right: hepatoblastoma and control cores

Criteria for p53 positivity

Cases were considered p53 positive when at least 10% of the tumour cells show nuclear positivity (score 2 or more). Staining in <10% of the tumour cells was considered as negative. Cytoplasmic staining was not accepted as positive.

Criteria for pRB positivity

A tumour was considered pRb positive when at least 10% of the tumour cells show nuclear staining (score 2 or more). Tumours were scored as pRb negative if <10% of the tumor cells show nuclear staining. Cytoplasmic staining was considered as negative.

Data analysis

The scoring and patterns of p53 and pRB expressions were analyzed to compare HCC against HB. Significance of association and correlation were based on the Fisher's exact test and Spearman rank correlation respectively.

Results

Demographic characteristics

26 HCC and 9 HB were included in the study. The demographic characteristics of patients are detailed in Table 1. The age range of HCC cases was 40-72 years with a mean of 57 years. The ages of HB cases ranged from 0.08 to 3 years, with a mean of 1.6 year. The majority of both HCC and HB cases were male and ethnic Chinese. It is noteworthy that 7 (27%) HCC cases were HbsAg positive. The hepatitis B viral status of HB cases was not known.

Histological typing

HCC cases comprised 3 well-differentiated tumours (Figure 2A) and 23 moderately-differentiated tumours. 6 HB exhibited a mesenchymal component (Figure 2B) and were classified as mixed hepatoblastomas. The remaining 3 HB exhibited pure epithelial component of the fetal subtype.

Table 1. Demographic Profile of Hepatocellular Carcinoma (HCC) and Hepatoblastoma (HB) Cases Studied

		HCC	HB
Number of cases		26	9
Male: Female		19:7	5: 4
Age range, years (Median, Mean)		40-72 (57, 56.8)	0.08-3 (1, 1.56)
Ethnic origin	Malay	3 (12%)	0
	Chinese	23 (88%)	9 (100%)
	Indian	0 (0%)	0
Hepatitis B surface Ag positive		7 (27%)	ND

*ND: Not determined

Table 2. Comparison of p53 and pRb Immuno histochemical Expression in 26 Hepatocellular Carcinoma (HCC) and 9 Hepatoblastoma (HB) Cases Studied

Immunohistochemical profile	HCC No. (%)	HB No. (%)
p53+, pRB+	1 (3.8)	0
p53+, pRB-	9 (34.6)	0
p53-, pRB+	1 (3.8)	1 (11.1)
p53-, pRB-	15 (57.7)	8 (88.8)
Total	26	9

p53 protein expressions

Table 2 summarises the immunopositivity rate for p53 and pRB proteins in the cases studied. p53 protein was detected in at least 10% of tumour nuclei in 10 (38%) of 26 HCC (Figure 3). The percentage of positive cells varied from 10-25% in 5 cases, 26-50% in 4 cases and >75% in one case. Of the cases regarded as p53-immunonegative, 4 showed nuclear staining in <10% of tumour cells while the 12 did not exhibit any staining.

All 9 HB cases were considered immunonegative for p53 protein. 5 HB cases showed no nuclear staining for p53 protein where 4 cases showed weak and focal nuclear staining in <10% of tumour cells.

Positive and negative control cores stained appropriately.

pRb protein expression

Two (7.7%) HCC cases were immunopositive for pRB and exhibited staining in 10-25% of tumour nuclei. 20 showed negative nuclear staining in all tumour cells and 4 cases showed weak and focal staining in <10% of the tumour cells.

One (11.1%) HB cases showed nuclear staining for pRB in at least 10% of tumour cells (Figure 4). 3 showed negative staining in all tumour cells while 5 showing weak and focal nuclear staining in <10% of the tumour cells.

Positive and negative control cores stained appropriately.

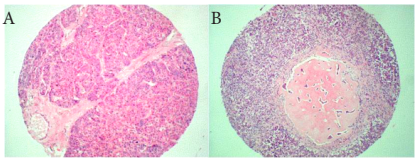

Figure 2. H&E Stained Tissue Cores x40. A) Hepatocellular carcinoma with trabecular pattern; B) Mixed hepatoblastoma showing peripheral epithelial and central mesenchymal elements

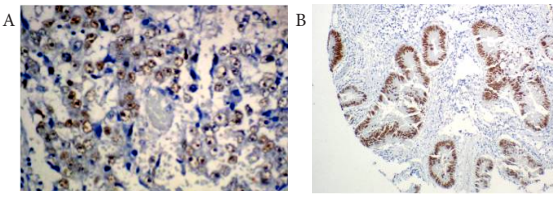

Figure 3. p53 Immunohistochemistry. A) HCC with positive nuclear staining (x200); B) Colonic adenocarcinoma (positive control) with positive nuclear staining (x80)

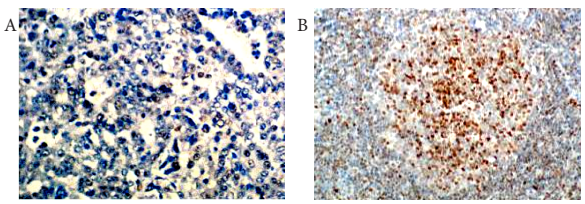

Figure 4. pRB Immunohistochemistry. A) HB with positive nuclear staining (x200); B) Tonsillar germinal centre (positive control) with positive nuclear staining (x100)

Statistical analysis did not reveal any significant association or correlation between the expressions of p53 and pRb in HCC and HB (Table 2).

Discussion

In this study, we used a standard immunohistochemical method for the assessment of p53 and pRb protein expresssion, as it is applicable on archival paraffin-embedded material. Because paraffin blocks are valuable for future studies and review, we elected to use tissue microarray to minimize tissue usage and to preserve as much as possible the original tissue blocks (Milanes-Yearsley et al., 2002).

There were minimal technical difficulties in creation of the microarray blocks. Each tissue core represented a 1mm diameter area of the selected tumour and contained approximately 500 to 1000 cells. The small area made counting and estimating the percentage of nuclear positivity easier, and reduced inter-observer variability. However, microarray poses a limitation on tumours with heterogeneous morphology and protein expressions. Taking this into consideration, we took two tissue cores from each tumour to reduce sampling error due to heterogenous morphology.

We observed that immunohistochemical nuclear staining for p53 and pRb were not confined to the tumour cells. p53 and pRb staining were also seen in the liver Kupffer cells and some inflammatory cells. This served as positive internal control in some of the tissue cores. However, most inflammatory cells did not stained for pRb protein. This casts some doubt over the negative pRb staining results of the HCC and HB cases. Nevertheless, the external positive control cores (tonsillar tissue) showed positive staining.

Our study revealed a p53 immunopositive rate of 38% in HCC. This finding is in agreement with other studies and suggests that p53 may have a significant role in HCC hepatocarcinogenesis (Choi et al., 1993; Schaff et al., 1995; Moudgil et al., 2013). However, mutational analysis will be required to confirm this suggestion, as a build-up of p53 protein as detected by immunohistochemistry is not necessarily indicative of mutation (Kennedy et al., 1994).

It is noteworthy that 3 of 10 HCC cases with p53 immunopositivity were also HBsAg positive. An association between p53 mutation in HCC and HBV infection has been suggested by other workers (Goldblum et al., 1993; Teramoto et al., 1994). Our findings are not against such an association, although the small number of cases does not permit a conclusion. p53 gene mutation at codon 249 and aflatoxin exposure in HCC has also reported (Hsu et al., 1991; Moudgil et al., 2013). Dietary exposure to aflatoxin has been noted to be a factor in Malaysian HCC, although we have no information regarding aflatoxin exposure in our study cases (Chen et al., 2004).

Cytoplasmic staining for p53 protein was observed in tumour cells in several cases. Although regarded as immunonegative, the significance of cytoplasmic localisation of p53 protein has not been fully explored. Generally regarded as a technical artifact, there have also been suggestions that it represents an attempt at conformational change of mutant p53 protein or binding to proteins of the heat shock protein family (Hagn et al., 2011).

All 9 HB cases in our study were negative for p53 immunohistochemistry. We note a study that showed p53 expression only in embryonal and small cell (dedifferentiated) subtypes of hepatoblastoma, and not in fetal or mesenchymal (well-differentiated) subtypes (Ruck et al., 1994). All our 9 HB tumours were pure epithelial (fetal type) or mixed (mesenchymal and fetal epithelial type). As the HB tumours in our study were relatively large and advanced at the time of resection, even a late build-up of p53 mutant protein would have been detected. Other studies have also reported a low positivity (of about 14%) for p53 by immunohistochemistry (Choi et al., 1993) suggesting that p53 mutation is a rare event in HB, and is probably not contributory to the development of HB. In this regard, it is noteworthy that hepatoblastoma is not a feature of the Li-Fraumeni syndrome which occurs as a result of TP53 germ-line mutation (Tomlinson and Kappler, 2012).

The Rb gene, located at chromosome 13q14, encodes a nuclear phosphoprotein pRb, which has a critical role in suppressing the G1 to S phase transition in cell proliferation control. Rb gene is deleted or mutated in a wide range of human cancers (Weinberg, 1995). Our study shows loss of pRb protein expression in the majority (24 cases, 92%) of HCC and (8 cases, 89%) of HB. Other studies on pRb immunohistochemical expression in HCC have yielded variable results with both loss of expression and high expression (Azechi et al., 2001; Huynh, 2004). This inconsistent expression may be linked to variable pRB function at different stages in the progression of the tumour (Hui et al., 1999). There is a scarcity of immunohistochemical studies investigating pRb protein expression in hepatoblastoma. A western immunoblot study showed HB and HCC cell lines with alterations in the Rb tumour suppressor gene in association with abnormal p53 expression (Farshid et al., 1994). In general, our findings are consistent with the concept that pRb functional loss occurs in HCC and HB carcinogenesis.

Comparative staining patterns for p53 and pRb proteins in our study did not reveal a useful staining profile to differentiate between HCC and HB. A predominantly double negative staining pattern was observed in the majority of both tumours (58% HCC and 89% HB) (Table 2). A smaller group (35%) of HCC cases was p53 positive and pRb negative while an even smaller percentage (11%) of HB was pRb positive and p53 negative. It would appear that, in the situation of a diagnostic difficulty in deciding between HCC and HB, a sensible consideration of the clinicopathological features of the case is more prudent than using immunohistochemistry.

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