Dissimilar Immunohistochemical Expression of ERK and AKT between Paired Biopsy and Hepatectomy Tissues of Hepatocellular Carcinoma

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Abstract. *Background/Aim: Biomarker studies using immunohistochemical (IHC) staining have not been successful for advanced hepatocellular carcinoma (HCC). We aimed to examine whether the tissue procurement process influences the protein expression levels detected by IHC. Materials and Methods: Forty-two tissue pairs of HCC that had been both preoperatively biopsied and then surgically resected were included in the study. IHC staining was used to determine expression of target molecules, all of which were graded according to the percentage of positively stained tumor cells. The expression of beta-catenin was analyzed according to the localization of positive staining. Results: Biopsied and surgically resected tissues exhibited dissimilar phosphorylated extracellular signal regulated kinase (p-ERK) and phosphorylated AKT expression levels (kappa=0.025 and 0.153, respectively). On the contrary, p53 exhibited similar expression levels, and beta-catenin exhibited similar staining localization patterns in biopsied and surgically resected tissues (kappa=0.729 and 0.654, respectively). Conclusion: Biopsied HCC tissues and their corresponding resected HCC tissues have inconsistent IHC-detected ERK and AKT expressions.*

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Immunohistochemical (IHC) staining is commonly used to detect biomarkers of diagnostic or prognostic importance in patients with malignant diseases. In some cancer types, the IHC-detected biomarkers also help direct treatment options. For example, patients with breast cancer are indicated for hormonal therapy if their tumor cells are stained positively for estrogen receptors (1).

However, prior IHC-based studies have not identified clinically useful biomarkers in the treatment of hepatocellular carcinoma (HCC). HCC is a malignant disease which is endemic in Asia and Africa and is gaining increasing importance in Western countries. Sorafenib, a multi-kinase inhibitor that targets RAF kinase and vascular endothelial growth factor receptor, is the current standard therapy for patients with locally advanced or metastatic HCC (2, 3). Several studies have used the IHC-detected expression of phosphorylated extracellular signal regulated kinase (p-ERK) as an indicator of the RAF/ERK pathway activity, and correlated this expression with efficacy of sorafenib in patients with HCC (4, 5). Unfortunately, these findings are conflicting and inconclusive.

Several technical factors during tissue procurement and processing may adversely affect the results of IHC studies that use archival formalin-fixed tissues. Protein stability is a critical issue. Proteins can degrade and/or lose their phosphorylation status, following prolonged intraoperative ischemia or in response to variable time frames preceding tissue fixation steps. In a study comparing phospho-AKT (p-AKT) expression levels in gastroesophageal cancer tissues, Baker *et al*. found that p-AKT expression was strong in most biopsy specimens but absent from all the surgically resected specimens (6). In a study on the stability of 53 signaling phosphoproteins, the expression level of several phosphoproteins varied by more than 20% within 90 min after tissue resection (7). The impact of this confounding factor on studies evaluating signaling proteins as biomarkers of HCC has never been addressed.

We hypothesized that the surgically resected HCC tissues, as a result of the complexity of the hepatectomy procedures, as well as variations in tissue processing methods, may not be suitable for studying the expression of certain protein markers using IHC staining. We designed this study to evaluate the consistency of expression analyses of several proteins, including the aforementioned p-ERK and p-AKT, in paired HCC tissues obtained from preoperative biopsy and surgical resection.

Materials and Methods

Study population. The data from patients who had undergone a hepatectomy for HCC at the National Taiwan University Hospital (NTUH), Taipei, Taiwan, R.O.C., from January 1, 2003 to December 31, 2008 were reviewed. Those with liver tumor biopsies within three months prior to hepatectomy were included in the current analysis. The archival biopsied and surgically resected specimens were retrieved. The study protocol was approved by the Institute Research Ethical Committee of NTUH, and all patients provided informed consent.

Immunohistochemical staining. Paraffin-embedded sections (5 μm in thickness) were de-paraffinized and then autoclaved in citrate buffer (pH 6.0) for 10 min using a decloaking chamber (Biocare, Concord, CA, USA) for antigen retrieval. They were incubated overnight at 4˚C with primary antibodies against p-ERK1/2 (Thr202/Tyr204) (1:100 dilution; Cell Signaling, Beverly, MA, USA), total ERK 1/2 (1:50 dilution; Cell Signaling), p-AKT (Ser473) (1:50 dilution; Cell Signaling), total AKT (1:100 dilution; Cell Signaling), p53 (1:200 dilution; Dako, Carpinteria, CA, USA), or beta-catenin (1:400 dilution; BD, Franklin Lakes, NJ, USA). Signal production employed adequate secondary antibodies, followed by horseradish peroxidase-labeled complex (Biocare Medical Starr Trek HRP Universal Kit; Biocare) and diaminobenzidine substrate. The slides were subsequently counterstained with hematoxylin.

All slides were blindly reviewed by a single pathologist (C-L Chen) unaware of the patient identity. Adequate positive controls (colon adenocarcinoma for p-ERK and p53; breast infiltrating ductal carcinoma for p-AKT and total ERK; lung adenocarcinoma for total AKT; and tonsil tissues for beta-catenin) were stained simultaneously to ensure staining quality.

Cytoplasmic staining of ERK and AKT, whether total or phosphorylated forms, and nuclear staining of p53 were graded according to the percentage of positively stained cells as negative: 0%; 1+: 1-9%; 2+: 10-50%, and 3+: >50%. The expression pattern of beta-catenin was analyzed according to the localization of positive staining, *i.e.* membranous, cytoplasmic, or nuclear staining.

Statistical analysis. Statistical analyses were performed with the SAS statistical software (version 9.1.3; The SAS Institute, Cary, NC, USA). Cohen's kappa coefficients were used to analyze the similarities of staining results between the biopsied and the resected HCC tissues. The relationship between kappa co-efficients and level of agreement followed Landis *et al*. (8): <0.00, no agreement; 0.00-0.20, slight agreement; 0.21-0.40, fair agreement; 0.41-0.60, moderate agreement; 0.61-0.80, substantial agreement; 0.81-1.00, almost perfect agreement.

Table I. *Dichotomized immunohistochemical staining results (low: 0 or 1+, high: 2+ or 3+) between biopsied and surgically resected hepatocellular carcinoma tissues. Staining results of beta-catenin was dichotomized into nuclear translocation or not.*

		Surgically resected		
Biopsied		Low	High	kappa
Phospho-ERK	Low	4	1	0.025
	High	26	10	
Total ERK	Low	7	1	0.205
	High	17	16	
Phospho-AKT	Low	5	1	0.153
	High	18	18	
Total AKT	Low	13	2	–0.043
	High	24	2	
p53	Low	26	1	0.729
	High	4	11	
Beta-catenin	Nuclear translocation	No	Yes	
	No	27	1	0.654
	Yes	5	9	

ERK=Phosphorylated extracellular signal regulated kinase. Low-expression=grades 0 or 1+; high-expression=grades 2+ or 3+.

Results

The paired HCC tissues of 42 patients were included for analysis. The expression levels of p-ERK, total ERK, p-AKT, total AKT and p53 were given semi-quantitative grades according to the percentage of positively-stained tumor cells (Figure 1A-E). The expression of beta-catenin was recorded according to the localization of positive staining (membranous, cytoplasmic, or nuclear staining) (Figure 1F). Because the biopsy sample in one patient was very small, p-ERK and total ERK results were available for only 41 patients.

Dissimilar ERK and AKT expression levels in paired HCC tissues. The p-ERK expression levels in paired biopsied and resected HCC tissues were only in slight agreement (kappa=0.105, Figure 1A). After dichotomization into low-expression (grades 0 and 1+) *versus* high-expression (grades 2+ and 3+), which was often used in prior research, the agreement of p-ERK expression levels between the paired tissues was even poorer (kappa=0.025, Table I). Generally, the expression levels were lower in resected than in biopsied tissues. The p-ERK expression was low in 73% of surgically resected tissues and 12% of biopsied tissues ($p<0.001$). Similarly, the total ERK expression levels in surgically resected tissues were generally lower than those in biopsied tissues, and the agreement of total ERK expression levels between paired tissues was poor (Figure 1B). When the total ERK expression was further analyzed as low *versus* high

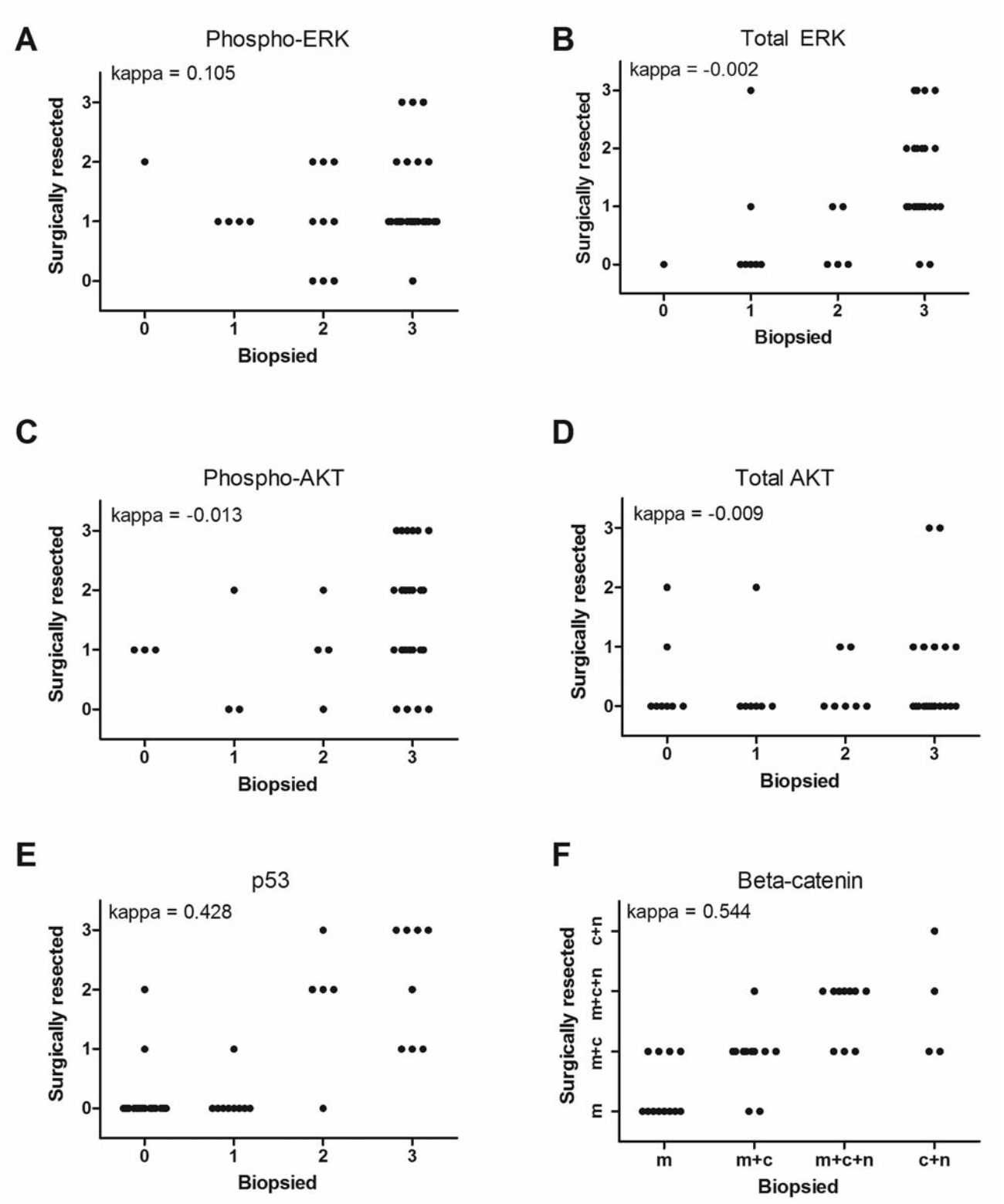

Figure 1. *Immunohistochemical staining results between biopsied and surgically resected hepatocellular carcinoma tissues, of (A) phosphorylated extracellular signal regulated kinase (phospho-ERK), (B) total ERK, (C) phospho-AKT, (D) total AKT, (E) p53, presented in grades according to the positive staining percentages; (F) beta-catenin, presented according to the location of positive staining (m=membranous, c=cytoplasmic, n=nuclear).*

expression, the agreement between paired tissues improved slightly but remained only in fair agreement (kappa=0.205). The p-AKT expression levels between paired biopsied and resected HCC tissues was in poor agreement (kappa=–0.013, Figure 1C). After dichotomizing into low-(grade 0 and 1+) *versus* high-(grade 2+ and 3+) expression, the p-AKT expression levels between paired tissues was only in slight agreement (kappa=0.153, Table I). Here again, p-AKT grades were generally lower in resected than biopsied tissues. The p-AKT expression was low in 55% of resected tissues and in 14% of biopsied tissues ($p<0.001$). The expression of the total AKT between paired biopsied and resected tissues showed no agreement whether analyzed before dichotomization (kappa=–0.009, Figure 1D) or after dichotomization (kappa=–0.043, Table I).

p53 and beta-Catenin expression similarity in paired HCC tissues. The expression levels of p53 were in moderate agreement between paired HCC tissues (kappa=0.428, Figure 1C). When p53 expression was analyzed as low *versus* high, only 5 out of 42 (12%) patients had discrepant results (Table I), and the kappa co-efficient showed substantial agreement between paired tissues (kappa=0.729).

The localization of positive staining of beta-catenin, especially the nuclear staining, is considered a reflection of activated WNT/beta-catenin pathway, and is widely studied for its associations with treatment outcomes of HCC (9, 10). We found that the expression patterns of beta-catenin staining between paired HCC tissues exhibited moderate agreement (kappa=0.544, Figure 1F). When the beta-catenin expression was analyzed as being with or without nuclear translocation, only 6 (14%) patients had differing results. The results between biopsied and resected tissues showed substantial agreement (kappa=0.654, Table I).

Case presentation. A representative case showing dissimilarity in p-ERK and p-AKT expression between paired biopsy and hepatectomy HCC tissues but similar p53 expression and beta-catenin location, is demonstrated in Figure 2. Both p-ERK and p-AKT expressions from tissues by biopsy were graded as 3+, but those from tissues by surgical resection were grade as 1+. On the contrary, p53 expressions of the biopsied and resected tissues were both graded as 2+. No nuclear translocation of beta-catenin was found in biopsied and resected tissues for this presented case.

Discussion

In the current study, we demonstrated that IHC-detected expression levels of ERK and AKT in HCC tissues were significantly different in paired HCC tissues obtained from preoperative biopsy and surgical resection. Both phosphorylated forms and total forms of ERK or AKT showed poor consistency in their expression patterns between paired tissues. Overall, the expressions of both proteins were significantly lower in surgically resected tissues than in their preoperatively biopsied counterparts. On the contrary, the expression of p53 and beta-catenin nuclear translocation were in much better agreement between paired HCC tissues. Differences in the protein expression levels detected by IHC staining as a result of varying tissue procurement procedures were reported for other types of cancer (6, 11). In gastroesophageal cancer, positive IHC staining of p-AKT was observed in 9 out of 13 tumor samples obtained by biopsy but not in any of the 15 samples obtained by surgical resection (6). In non-small cell lung cancer (NSCLC), the levels of p-AKT and epidermal growth factor receptor (EGFR), detected by IHC staining, were significantly lower in surgically resected tissues than in their biopsied counterparts (11). Together with our findings, these data imply that surgically resected tissues, because of tissue injury during the surgical procedures and differences in tissue processing procedures postoperatively, may not be reliable for assessment of certain biologically active markers.

The mechanism underlying the inconsistency of IHC-detected ERK and AKT expression between biopsied and resected HCC tissues is multifactorial. Degradation, as well as de-phosphorylation, of ERK and AKT may occur during surgical and subsequent tissue processing procedures. Hepatectomy is a complicated surgical procedure, involving different degrees of blood-flow interruption by arterial occlusion (12, 13). Both tissue ischemia and surgical stress may alter the expression of signaling pathway molecules. The time-to-fixation is also significantly different between biopsied and surgically resected tissues. The time frame between biopsy and adequate fixation is likely to be rapid, while that between hepatectomy and fixation is generally delayed and highly variable. Mass spectrometry has detected changes in the expressions of a significant portion of proteins in as short as 10 minutes after specimen resection (14). We and others have also demonstrated a rapid decline in expression of p-ERK and p-AKT within 30 min after tumor resection (6, 15).

The p53 levels and the nuclear translocation of beta-catenin, detected by IHC were relatively consistent between the biopsied and resected HCC tissues. These results, together with the ERK and AKT data, indicate that the impact of tissue procurement and tissue processing could differ among proteins. In the report of Taillade *et al*., which showed differences in levels of p-AKT and EGFR between paired NSCLC samples, the levels of excision repair cross complementing (ERCC)1, human telomerase reverse transcriptase (hTERT), and Ki-67, were relatively stable (11).

Our findings raise the issue about which types of HCC tissues should be used for IHC studies. In prior biomarker studies of HCC, biopsy and surgical specimens were

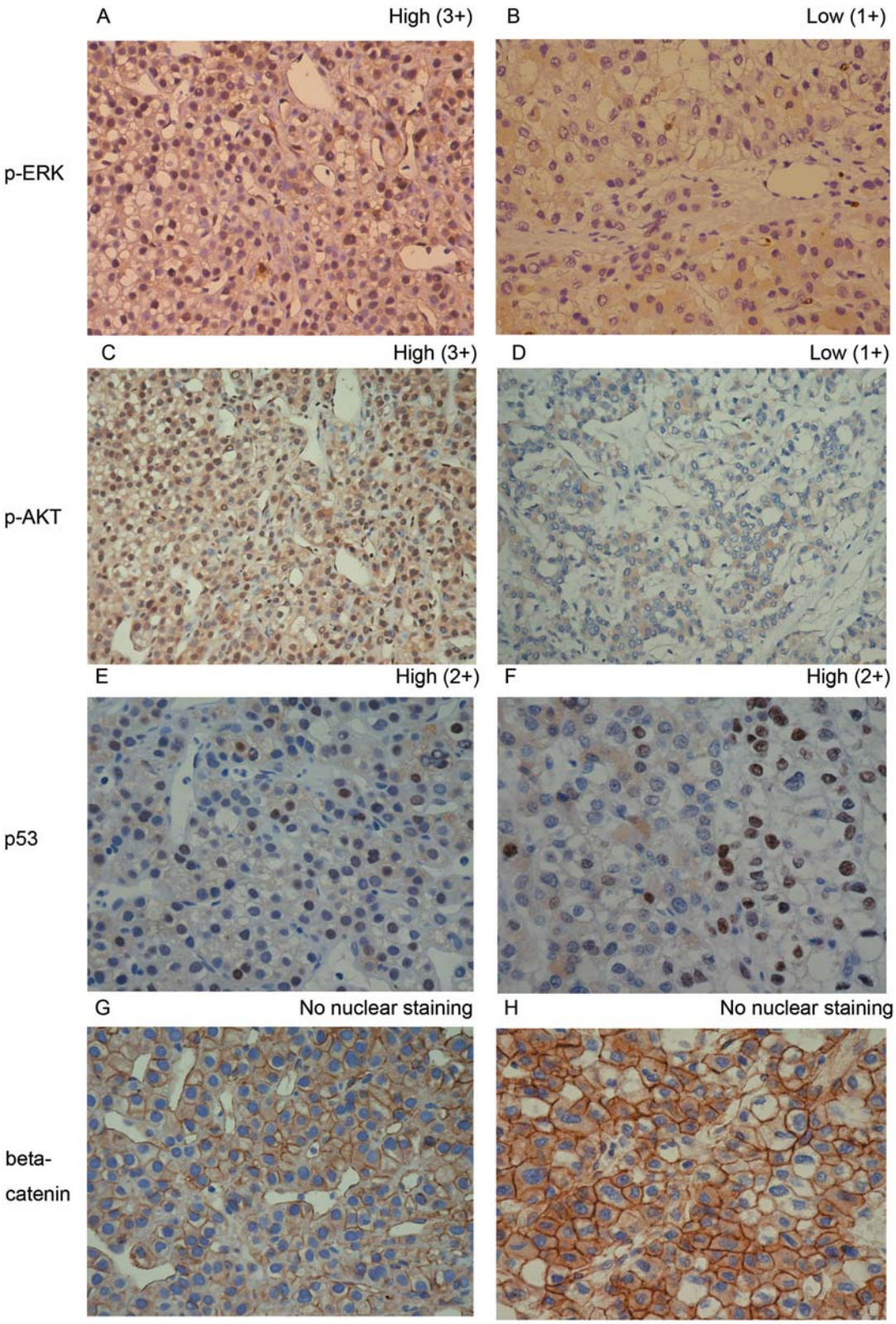

Figure 2. *Immunohistochemical staining results of HCC tissues from biopsy or hepatectcomy of a representative case. (A, B) Phosphorylated extracellular signal regulated kinase (phospho-ERK); (C, D) phospho-Akt; (E, F) p53; (G, H) beta-catenin; (A, C, E, G) tissues from biopsy; (B, D, F, H) tissues from surgical resection.*

obtained at variable durations before the treatment started. As shown in this study, the inconsistency of IHC results between tissues obtained by different procurement procedures may result in systematic bias. To avoid this confounding factor, similarities in biomarker expression between biopsied and resected specimens should be confirmed before including both types of tissue sources for IHC analysis.

In conclusion, IHC-detected expression levels of ERK and AKT are significantly lower in resected HCC tissues than in their preoperatively-biopsied counterparts. Caution should be used when combining biopsied and post-hepatectomy samples while studying the expression levels of these and other protein markers.

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