Clinical Studies

Proinflammatory Homeobox Gene, *ISX*, Regulates Tumor Growth and Survival in Hepatocellular Carcinoma

Shih-Hsien Hsu1, Li-Ting Wang1, King-Teh Lee3,4, Yao-Li Chen5,7, Kwei-Yan Liu1, Jau-Ling Suen1, Chee-Yin Chai2, and Shen-Nien Wang1,4,5

Abstract

Chronic inflammation drives initiation of hepatocellular carcinoma (HCC), but the underlying mechanisms linking inflammation and tumor formation remain obscure. In this study, we compared the expression of interleukin (IL)-6 and cyclin D1 (CCND1) with the IL-6-induced homeobox gene *ISX* (intestine-specific homeobox) in 119 paired specimens of HCCs and adjacent normal tissues and also in paired specimens from 11 patients with non-HCCs. In pathologic analysis, ISX exhibited a tumor-specific expression pattern and a high correlation to patient survival time, tumor size, tumor number, and progression stage. Enforced expression of ISX accelerated cell proliferation and tumorigenic activity in hepatoma cells through CCND1 induction. In contrast, short hairpin RNA-mediated attenuation of ISX in hepatoma cells decreased cell proliferation and malignant transformation in vitro and in vivo. A high positive correlation existed in human hepatoma tumors between ISX and CCND1 expression. Together, our results highlight ISX as an important regulator in hepatoma progression with significant potential as a prognostic and therapeutic target in HCCs.

Cancer Res; 73(2); 508–18. ©2012 AACR.

Introduction

Hepatocellular carcinoma (HCC), the fifth most commonly occurring cancer and the third leading cause of cancer-related deaths every year worldwide, has a multiple step progression with a high evidence of association with chronic inflammation exposure induced by environmental toxin intake and/or viral infection, such as HBV or HCV (1). The chronic inflammation often interacts with innate and adaptive immune responses, and the detailed regulatory mechanism leading to HCC tumor formation is unclear to date.

D-type cyclins are the major regulators governing G1 progression to S-phase in response to mitogenic and oncogenic signals and serve as markers for human malignancies (2, 3). Through binding with and activation of their associated cyclin-dependent kinases, CDK4 and CDK6, cyclin D–CDK complexes phosphorylate the retinoblastoma tumor suppressor gene products, pRB, and pRB-related proteins, p130 and p107 (4, 5). This phosphorylation aborts growth-inhibitory functions of pRB, which leads to release of the E2F1 transcription factors and allows induction of E2F1 target genes that are required for progressing into S-phase (6). The growth-promoting functions and deregulation expression of D cyclins is a driving force toward increased tumor proliferation and transforming activity in several human cancers (2, 7). D cyclin overexpression in human cancers is driven by several mechanisms including transcriptional activation, genome alteration, posttranscriptional regulation, and posttranslational protein stabilization (2, 3). Furthermore, several mouse models have also shown that cyclin D1 can cooperate with other oncogenes to transform cells (8, 9). The oncogenic characterization of cyclin D1 explored in cancer models and human cancers suggests that cyclin D1 and its associated CDKs activity may be a potential therapeutic target (3).

Homeobox genes, a superfAMILY of transcription factors with homeo domains, play an essential role in controlling cell growth, differentiation, and morphogenesis during early embryonic development (10). Deregluation of homebox genes appears to increase cell survival and proliferation and inhibit cell differentiation (11, 12). Recently, many homebox genes were found to be aberrantly expressed in a wide variety of human tumor masses (13). Intestine-specific homebox (Isx) is a newly identified pair family homeobox transcription factor, which shows a intestine-specific expression pattern in both adult and fetal intestines (14). Phylogenetic analysis showed that the homeo domain of Isx...
Ixsx is an ectopically expressed homeobox gene found in hepatoma tumor cell lines and tumor masses. The cellular function and tumor-specific expression pattern suggests that Ixsx is an important activator in proliferation and tumor formation in HCCs.

Materials and Methods

Patients

This study assessed a total of 123 patients with HCCs from July 2004 to November 2009 from multiple medical centers [Chung Ho Memory Hospital (89 HCCs) and Changhua Christian Hospital (34 HCCs)] with HBV and/or HCV infection and 11 patients with non-HCCs (4 infected with HBV/HCV and 7 non-infected with HBV or HCV) were enrolled into the Ixsx study, 119 of whom had adequate follow-up data for analysis. Experiments of human samples have been approved by Institutional Review Boards of Kaohsiung Medical University (Kaohsiung, Taiwan; ref. 15).

Plasmids and cell lines

Full-length Ixsx cDNA was PCR-amplified from a human placenta cDNA library (GIBCO/BRL) and different truncated Ixsx cDNAs were subcloned into the pEGFP/C1 vector (Clonetech) to express the GFP-tagged Ixsx protein. PGI2PZ was used for Ixsx short hairpin RNA interference (shRNAi) construction. The following sequences were used to construct Ixsx shRNAs: shRNAi-1(1031-1051): 5’-TGAGCCTGTCCTTTTCCTCA-3’ and shRNAi-2(1367-1387): 5’-AGCACGAGAGATTTGACACCC-3’. HEK 293, Hep G2, PLC5, HA22T, SK-Hep1, Huh7, and Hep 3B cell lines were subcultured and maintained according to ATCC protocol. Transfection was conducted using the Lipofectamine Transfection Kit (GIBCO/BRL).

Western blot, luciferase reporter assays, and immunohistochemical staining analysis

Western blot, luciferase reporter assays, and immunohistochemical (fluorescence) staining analysis were done as previously described (15).

Quantitative PCR, chromatin immunoprecipitation, and EMSA

The above assays were analyzed as previous described (15).

Tumorigenic assay of Ixsx in soft agar and nude mice assay

Cells (10^4 or 5 × 10^3) in 1-mL culture medium were mixed with an equal volume of 0.6% of top agar and plated onto 60-mm dishes with 0.5% bottom agar (15). More detail processes could be found in Supplementary Methods and Materials.

Statistical analysis

The quantitative variables are presented as means and SDs. Statistical differences were determined with 2-sample t test. Pearson correlations were used to determine the correlation coefficient between expression levels of Ixsx and cyclin D1. Statistical analysis of categorical variables was carried out using χ² analysis, one-way ANOVA, and Fisher exact analysis. P < 0.05 was considered statistically significant.

Results

Patient characteristics

Ixsx is an ectopically expressed homeobox gene found in hepatoma microarray analysis (16); however, the correlation between clinical outcome of HCCs and cellular function of Ixsx is unknown to date. To characterize the regulatory effect and clinical outcome of Ixsx in HCCs, 123 patients with HCCs from 2 medical centers were enrolled into the Ixsx cohort study from July 2004 to November 2009. Of these, 119 had adequate follow-up data for analysis. The baseline characteristics of patients with HCCs and non-HCCs were compared between groups with non-HCCs, low and high Ixsx expression, and the results are shown in Table 1. The overall survival time of all patients was 48 months. There were significant associations in terms of albumin (P = 0.0040), bilirubin (P = 0.0282), asc sugar (P = 0.0281), tumor size (P < 0.0001), number of tumors (P < 0.0001), and tumor grade (P < 0.0001), but not in age, sex, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, α-fetoprotein, γ-glutamyltranspeptidase (γ-GT), cholesterol, and triglycerides (Table 1).

The Ixsx expression pattern was then detected in HCC tumor samples by immunohistochemistry staining with anti-Ixsx polyclonal antibody (Fig. 1A). Ixsx (brown, yellow arrows) showed a tumor-specific expression pattern in HCC tumor mass (left) compared with the adjacent normal liver tissue (black star) and was detected in both cytoplasm (red arrow) and nuclei (blank arrow) in tumor cells (right). Ixsx mRNA expression in HCC liver samples (81 patients with high Ixsx expression and 38 patients with low Ixsx expression) was significantly upregulated compared with patients with non-HCCs analyzed with one-way ANOVA (Fig. 1B, P < 0.001). Analysis of the survival curves of patients with HCC found that patients with HCCs with lower Ixsx expression had a significantly longer survival time than those with higher Ixsx expression after liver excision surgery (Fig. 1C, P = 0.0238). Ixsx expression seems to correlate with the expression of cyclin D1, an important cell-cycle regulator. Three HCC tumor species (with HBV and HCV infection) and 6 different hepatoma cell lines were found to have high ectopic Ixsx protein expression than normal liver tissue (with no HBV/HCV infection; Fig. 1D). The Ixsx and cyclin D1 mRNA expression in HCC tumor specimens from 99 patients with HCCs and 11 non-HCC liver biopsies showed that the Ixsx mRNA expression was highly associated with cyclin D1 expression in all patients (R = 0.7415, P < 0.0001; Fig. 1E). These results suggest that Ixsx plays an important regulatory role in HCCs,
Table 1. Baseline characteristics of 119 patients with HCCs compared with 11 patients with non-HCCs

<table>
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<th>Group</th>
<th>Non-HCC (n = 11)</th>
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<th>Isx mRNA (&gt;3; n = 81)</th>
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<td>Age [mean(SD)]</td>
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NOTE: P values were calculated by Fisher exact tests. Patients: 123 patients with HCC from 2 medical centers [Chung Ho Memorial Hospital (89 HCC) and Changhua Christian Hospital (34 HCC)] with HBV and/or HCV infection and 11 non-HCCs.

*Non-detectable data of patients from Changhua Christian Hospital.

*P values were calculated by Fisher exact test.

*P < 0.05.
progression and patients survival. The highly correlated expression between Isx and cyclin D1 also suggests that Isx could be a predictive marker for cyclin D1 expression and hepatoma growth.

**Proinflammatory cytokines induced Isx expression through NF-κB signaling pathway**

To explore the association of Isx expression with inflammatory cytokines in HCC tumor formation, the proinflammatory cytokines elevated in serum or liver tissue of patients with hepatoma in previous studies (17–19) were first used to treat 6 HCC cells (Hep G2, Hep 3B, SK-Hep1, Huh7, PLC/PRF/5, and HA22T, HCC tumor cell lines). E, the mRNA expression of cyclin D1 in non-HCC and HCC tumor species showed highly correlated expression with Isx mRNA expression.
showed that IL6 transcriptionally activated Isx expression through the activation of NF-κB signaling.

The transcripational regulatory elements induced by IL6 on Isx promoter (Fig. 3A) were then analyzed by promoter assay and chromatin immunoprecipitation (ChIP). IL6 significantly increased Isx promoter–driven luciferase activity (3.945 ± 253) until the promoter sequence had been deleted to a length shorter than 220 bp (Fig. 3B). Different segments of oligonucleotides between −320 and −220 bp in the Isx promoter region were then synthesized to determine the NF-κB (p65) binding affinity by electrophoresis mobility shift assay (EMSA) in vitro. The nuclear proteins of IL6-treated cells showed high binding affinity and were supershifted by the addition of an anti-p65 antibody in the −294 to −318 bp region on the Isx promoter (Fig. 3C). Deletion of this NF-κB binding element showed abotion of the luciferase activity induced by IL6 (Fig. 3B). Furthermore, the Isx promoter region (−320 to −220 bp) could be pulled down (3.91-fold) in p65 (Rel A) immunoprecipitates treated with IL6 compared with vehicle-treated cells (Fig. 3D). This promoter-binding activity of p65 induced by IL-6 was abated when the cells were treated with NF-κB–specific inhibitor, BAY 11-7085 (5 μmol/L), MAPK kinase inhibitor (U0126, 5 μmol/L), and JAK/STAT2 inhibitor (AG490, 20 μmol/L). These results suggested that signals (MAPK and JAK/STAT2) activated by IL6 could activate Isx mRNA expression through increasing NF-κB (p65) binding activity on Isx promoter. However, NF-κB signaling activated by IL6 regulated Isx expression at both the transcriptional and translational level.

Moreover, dietary vitamin A intake has been shown to regulate intestinal Isx expression (3), and its metabolites are suggested to play a role in tumorigenesis. We next examined the potential role of vitamin A and its related metabolites on IL6–induced Isx expression. Higher levels of retinoid X receptor (RXR) binding to the Isx promoter were detected in hepatoma cells than those noted in normal hepatocytes that showed no detectable expression of Isx (Fig. 3E). Interestingly, while IL6 treatment increased Isx expression in hepatoma cells, the levels of the binding of RXRα to the Isx promoter were significantly reduced (ranging from 52% to 58%) in IL6-treated cells compared with vehicle-treated cells. (Fig. 3E). These results suggested that signals (MAPK and JAK/STAT2) activated by IL6 could activate Isx mRNA expression through increasing NF-κB (p65) binding activity on Isx promoter. However, NF-κB signaling activated by IL6 regulated Isx expression at both the transcriptional and translational level.

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hepatocytes, SK-Hep1 cells and, to a lesser degree, in Hep G2 cells (18% reduction; Fig. 3F). These results suggested that the Isx expression induced by IL6 was a retinoid-independent regulatory pathway.

**Isx expression enhanced cell proliferation in HCC cell lines**

To characterize the cellular function of Isx, wild Isx and different truncated proteins tagged with GFP were transfected and characterized in Hep G2 cells. Overexpressed Isx protein (green) was mainly detected in nuclei (blue), but Isx protein with the deleted homeobox domain was detected in whole cells including cytoplasm and nuclei, with loss of its original nuclear localization (Fig. 4A). Interestingly, the GFP protein fused with the Isx homeobox domain showed a nuclear translocation pattern similar to that of the wild Isx protein (Fig. 4A). The cell proliferation activity of Isx in Hep G2 cells was then determined with cell growth curves and [H³] thymidine incorporation analysis. Hep G2 cells transfected with Isx or GFP with Isx homeo domain showed a higher proliferation rate than that of cells transfected with mock only or Isx protein without the homeo domain (Fig. 4B). Also, overexpressed Isx increased [H³] thymidine incorporation activity 4.9-fold in comparison to mock-transfected Hep G2 cells after 16 hours incubation (Fig. 4C). The increase in proliferation activity induced by Isx was further observed in cell-cycle analysis with flow cytometry. The Hep G2 cells transfected with Isx showed an increase in sub-G₁ (1.6%–4.2%) and S-phase cell-cycle population (20.3%–30.5%) and a decrease in G₁ (42.8%–39.6%) and G₂–M (25.7%–18.9%) cell-cycle population compared with mock-transfected only (Fig. 4D). These results suggested that Isx increased cell proliferation to speed up cell-cycle progression from G₁ to S-phase despite an increase in sub-G₁ apoptotic cells.

**Isx upregulated cyclin D1 and E2F1 expression in HCC cells**

To determine the direct targets regulated by Isx, established regulators of the G₁–S transition were monitored by Western blot analysis. As shown in Fig. 5A, overexpression of Isx in Hep G2 cells increased D cyclins (1 and 3) and E2F1 protein expression and cells transfected with Isx containing a deleted...
Isx expression decreased to the mock-transfected level when promoter and the upregulated luciferase activity induced by Isx upregulated the luciferase activity driven by the cyclin D1 regulatory activation effects of Isx. As shown in Fig. 5D (bottom), and transfected into Hep G2 cells to evaluate the transcriptional activation effects of Isx. This result further confirmed that transcriptional regulation of Isx on cyclin D1 promoter was through the binding sequence between −260 and −297 bp.

Isx increased cyclin D1 expression through directly binding to the cyclin D1 promoter in vitro and in vivo

To further delineate the regulatory elements of Isx on the cyclin D1 promoter region, the different regions of oligonucleotide between −260 and −297 bp in cyclin D1 promoter were synthesized for EMSA analysis in vitro. The nuclear proteins extracted from Hep G2 cells transfected with Isx could specifically bind to the region between −260 and −272 bp, and the binding complex also showed a super shift (arrow) of the anti-Isx antibody in vivo (Fig. 5E). The cyclin D1 promoter fragment (−197 to −330 bp) bonded by Isx was detected by ChIP assay in vivo and showed an increase up to 11.6-fold compared with mock-transfected cells (Fig. 5F). The binding activity of Isx on cyclin D1 promoter abated after NF-κB inhibitor (BAY 11-7085, 5 μmol/L) treatment (Fig. 5F). Interestingly, MAPK (ERK1/2) inhibitor (PD98059, 50 μmol/L) also showed a decrease to baseline on the cyclin D1 promoter binding activity of Isx (Fig. 5F). The regulatory effect of Isx on cyclin D1 was also determined in patients with HCCs. Isx (green) showed highly expression localization with cyclin D1 (red) and Ki67 (pink), a cell proliferation marker. These results showed that Isx directly bound to cyclin D1 promoter and activated cyclin D1 (E2F1) expression transcriptionally.

Isx expression was essential for cyclin D1 expression and proliferation activity in HCC cells

To further evaluate the essential role of Isx on cell proliferation and tumorigenic activity, 2 sequence-specific shRNAi...
of Isx were transfected into 4 types of hepatoma cells with ectopic Isx expression (Hep G2, Hep 3B, SK-Hep1, and HA22T) and the knockdown efficiency in these hepatoma cells was examined by Western blotting (Supplementary Fig. S3 and Fig. 6A). Isx protein expression was decreased 85% in Hep G2 cells but the knockdown was less efficient in Hep 3B cells. These hepatoma cells with Isx knockdown were first examined to determine the cell proliferation activity by [H3] thymidine incorporation assay. The [H3] thymidine incorporation rate in Isx knocked down cells showed a significant decrease of 62%.

Figure 5. Isx increased cyclin D1 expression through direct promoter binding. A, overexpressed Isx increased the expression of cyclin D1 and E2F1. Arrowhead, truncated Isx expression. B, Isx expression increased cyclin D1 and E2F1 expression in hepatoma cells. Arrow, E2F1. C, Isx transcriptionally activated cyclin D1 and E2F1 promoter activity. ***, P < 0.001. D, Isx transcriptionally activated cyclin D1 promoter activity. Top, series of deletion constructs of cyclin D1 promoter. Bottom, relative luciferase activity. E, Isx nuclear extract from Hep G2 cells specifically binds the oligonucleotides of the cyclin D1 promoter region (–260 to –272 bp) in vitro. The DNA-binding activity of Isx nuclear extract was analyzed with EMSA. F, Isx directly bound to the promoter region of cyclin D1 determined with ChIP assay in vivo. Top, agarose gel electrophoresis analysis of ChIP. Arrowhead, cyclin D1 promoter region (–197 to –330 bp); positive, 10% of input control; negative, no antibody. Bottom, the quantitative statistical analysis of cyclin D1 promoter–binding activity of Isx was determined by ChIP assay. ***, P < 0.001, compared with Isx-transfected only (n = 3, means ± SD).
56%, 54%, and 68%, respectively, in thymidine incorporation activity compared with those that were mock-transfected (Fig. 6B). Furthermore, many G1–S transition regulators, such as cyclin D1 (84%), CDK4 (62%), and E2F1 (86%), were significantly downregulated in Isx shRNAi cells (Fig. 6C and Supplementary Fig. S4). Some apoptotic factors were also shown to be elevated in Isx shRNAi cells (Supplementary Fig. S5). Other tumor suppressors, such as p19 and p21, did not show any regulatory effect.

Isx expression was critical for hepatoma tumorigenic activity

Following the determination of cellular proliferation, the transformational and tumorigenic activity of Isx shRNAi cells were then determined with a foci formation on soft agar anchorage-independent assay in vitro and tumor growth in nude mice in vivo. As shown in Fig. 6D, over-expression of Isx increased the transforming activity (54%) in Isx-transfected cells compared with mock-transfected Hep G2 cells; however, Isx shRNAi cells showed a significant decrease in transforming activity (84%) in comparison with mock-transfected cells in vitro. Furthermore, Hep G2 (Fig. 6E) and Hep 3B cells (Fig. 6F) with overexpressed Isx showed an increase in tumor size and tumor growth activity in nude mice in vivo compared with mock-transfected cells, and Isx shRNAi cells showed a significant decrease in tumor size and tumor growth activity compared with mock-transfected cells. These results suggest that Isx plays an essential role in promoting the transforming and tumorigenic activity of hepatoma cells.

Discussion

In this study, we identified Isx as a proto-oncogenic homeobox gene specifically overexpressed in HCC tumor cells. Isx, an IL6-induced homeobox gene, upregulated cyclin D1 and E2F1 expression through the NF-κB signaling pathway. Through direct binding to the cyclin D1 promoter, Isx increased cellular cyclin D1 expression through which it regulated the cell proliferation and transforming activity of HCC tumor cells in vitro and in vivo. The tumor-specific expression pattern,
highly correlated to survival time, tumor progression, and cyclin D1 (IL-6) expression in patients with HCCs, suggested that Isx plays an essential role in hepatoma tumor formation and prognosis.

HCC is a chronic liver disease that occurs as a multistep process characterized by progressive accumulation of genetic alterations causing aberrant growth, malignant transformation, and metastasis (21). Homeobox genes, essential regulatory transcription factors for multiple body plan development (10), have been found to be deregulated in many malignancies and are thought to be potential oncogenes (11). Few homeobox genes have been found to be involved in HCC development, excluding Hox, Prox1, and CDX2; however, most of them are expressed both in normal and in tumor cells and are thought to act as tumor suppressors in the liver (22–24). In this study, we found Isx, in particular, ectopically expressed in tumor cells of HCC tumor mass, but not normal cells, and highly correlated to cyclin D1 expression in HCC tumor cells. This tumor-specific pattern and the gene silencing results suggest that Isx is an important proto-oncogenic protein in HCC development. Isx, a gut-specific transcription factor, was a putative repressor for intestinal SREB and BCMOI expression that consequently repressed vitamin A production and downstream retinoic acid (RA) signaling (3, 14). Retinoid-induced signaling is important in regulating cell growth, differentiation, and development (25) and found to abnormally express and highly associated with tumor development in many malignancies, including HCCs (26). Deficiency in retinol and RXRα expression was found in tumor cells, but not in the adjacent normal hepatocytes in patients and animals with HCCs, which was adverse to Isx expression in patients with HCCs (27).

Also, our study suggested that IL6-induced Isx expression in hepatoma cells was independent of the retinoid signaling. These results appeared to be at variance with the previous studies showing that dietary vitamin A intake was able to increase intestinal Isx expression (3). IL6 might regulate vitamin A metabolism via Isx expression, but this is, at present, uncertain in the case of HCCs. More studies would be needed to elucidate the regulatory effect. Furthermore, the regulatory activity on vitamin A by ectopic Isx expression remains to be elucidated.

In Isx−/− mice, the cell growth regulatory and transforming activity were not observed in tissue development and the offspring were born in the expected numbers and appeared healthy for at least 1 year (14). This regulatory effect of Isx on proliferation and the tumor-specific ectopic expression pattern in HCC tumor cells suggests a malignant genetic alteration profile induced by chronic inflammation exists in HCC tumor cells. This malignant alteration circumvents the activating JAK/STAT3 signaling, NF-κB, and the JAK/STAT5 signaling, NF-xB and downstream genes, such as Isx, are consequently activated to mediate cell survival and G1 to S cell-cycle transition. In this study, one potential IkB-binding site (RNNYYCC) on the Isx promoter region (−294 to −318 bp) was found to respond to NF-xB regulatory activation in Isx expression. The results of this element deletion on the Isx promoter showed that this potential IkB element was essential to NF-xB activation. Also, from the mouse model, increased IL6 production has also been implicated in the pathogenesis of HCCs (32). Furthermore, in liver regeneration, IL6 regulates cellular proliferation through the activation of cyclin D1 expression in hepatocytes, which provides a model for cyclin D1 activation by proinflammatory cytokines in HCC development (33).

In summary, in this study, we found that Isx was an important tumor-specific proto-oncogenic homeobox gene. Through direct regulation of cyclin D1 and E2F1 expression, Isx regulated the proliferation of tumor cells and their transforming activity, and it appears worthwhile to further investigate its cellular function in HCCs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S.-H. Hsu, K.-T. Lee, K.-W. Liu, S.-N. Wang
Development of methodology: S.-H. Hsu, L.-T. Wang, S.-N. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.-T. Wang, Y.-L. Chen, K.-W. Liu, J.-L. Suen, C.-Y. Chai, S.-N. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.-H. Hsu, L.-T. Wang, K.-W. Liu, S.-N. Wang
Writing, review, and/or revision of the manuscript: S.-H. Hsu, S.-N. Wang

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Cancer Res; 73(2) January 15, 2013 517

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.-L. Chen, J.-L. Suen
Study supervision: S.-H. Hsu, K.-T. Lee, S.-N. Wang

Grant Support
This work was supported, in part, by research grant KMUH100-0820, KMUH100-0821, KMUER007-4, and NSC-101-2320-B-037-042 from the National Science Council, Taiwan.

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