miR-22 promotes HBV related hepatocellular carcinoma development in males

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Running title: IL-1 alpha regulates hepatocellular carcinoma
Application of this study toward the future practice of cancer medicine:

1) IL-1α is an essential factor for the development of HBV related HCC in males; moreover, it is able to specifically predict the direction of chronic liver inflammation. IL-1α could potentially be used as a marker in the clinical diagnosis of HBV related HCC.

2) Upregulation of IL-1α is associated with decreased ERα and increased miR-22 expression in adjacent human male tumor tissue, and these two indices may also be valuable markers in the clinical diagnosis of HBV related HCC.

3) Dynamic monitoring of IL-1α is also a potentially valuable tool in predicting HBV-associated HCC recurrence. It is important to employ suitable methods to prevent HCC recurrence and to improve survival.

4) IL-1α expression is also a potential target for pharmaceutical research.

Abstract

Purpose: Previous reports have demonstrated that IL-1α-MyD88-IL-6 signaling is essential in promoting hepatocellular carcinoma (HCC) development in a diethylnitrosamine (DEN)-induced mouse model. We aimed to determine whether IL-1α regulates HCC development in humans. Methods: HBV-associated HCC tissue, corresponding adjacent tissue, and normal tissue samples were obtained from 80 male and 36 female patients. IL-1α, ERα, IL-6, and MyD88 were quantified using real-time PCR and Western blot. Stem-loop PCR was used to quantify miR-22 expression. Luciferase reporter assays were used to study transcriptional regulation. Results: IL-1α was highly expressed in male tumor adjacent tissue compared to normal tissue (P=0.025); however, this was not the case for female subjects. A linear relationship was observed between increased IL-1α and decreased ERα expression
in male tumor adjacent tissue (R=-0.616, P=0.004). Our results also indicated that estrogen (E2) was suppressed upon IL-1α secretion in ERα overexpressed HCC cells. We detected high expression of miR-22 in male tumor adjacent tissue compared with controls (P=0.032); furthermore, we demonstrated that miR-22 downregulates ERα transcription by targeting the 3′UTR region. In the DEN-induced model, IL-1α was highly expressed in sprouting tumors and gradually decreased in conjunction with HCC development. **Conclusion:** Overexpression of miR-22 in male tumor adjacent tissue was associated with down-regulated ERα expression, potentially by attenuating the protective effect of estrogen and causing increased IL-1α expression. These results may explain the high incidence of HBV-associated HCC in the male population.

**Key words:** IL-1α; ERα; miR-22; hepatocellular carcinoma
Introduction

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver often secondary to a viral hepatitis infection (hepatitis B or C) or cirrhosis. Thus, hepatitis-related cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β), have been implicated in HCC development. Numerous previous studies have examined the effects of these cytokines upon the initiation, promotion, and progression of HCC (1-6).

Recent investigations have reported a relationship between inflammation and HCC in mouse models (7). These studies demonstrated the multi-faceted role of the NF-κB signaling pathway in HCC development. NF-κB maintains normal hepatic function by stimulating hepatocyte regeneration while also promoting neoplastic effects, such as cell survival (anti-apoptosis), angiogenesis, and proliferation in both precancerous and cancerous cells (8-10). Within this context, IL-1β-MyD88-IL-6 signaling is considered a leading factor in the diethylnitrosamine (DEN)-induced HCC mouse model. IL-1β has been shown to be upregulated during reactive oxygenated species (ROS)-induced necrosis in hepatocytes, and this in turn upregulates IL-6 expression via the MyD88 mediated signaling pathway in Kupffer cells (KCs). Previous reports have demonstrated that IL-6 overexpression can lead to HCC tumorigenesis in mice (11, 12). However, estrogen and estrogen-mediated signaling blocked IL-6 expression in KCs through the toll-like receptor adaptor protein MyD88, resulting in decreased HCC incidence in the DEN mouse model (12). This finding is a potential explanation for the sex disparity observed in HCC in both mice and humans.

Despite the extensive previous research performed in this subject area, very few investigators have examined IL-1α expression in human tumor specimens. Therefore, our objective was to determine whether the effects observed in a mouse model could be reproduced in human samples and to
examine whether other factors affect this signaling pathway. Additionally, we aimed to investigate IL-1α-MyD88-IL-6 signaling in patients with HCC and in patients with chronic hepatitis, a well-established precancerous state of HCC. These findings may provide further insight into the use of IL-1α as a therapeutic target for HCC treatment.

Materials and Methods

Patients

HCC and adjacent tissues were obtained from 80 male and 36 female patients at the time of surgical resection at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China) between January 2001 and August 2009. Normal liver parenchymal tissue was obtained from 48 patients, 28 male and 20 female (12 premenopausal and eight post-menopausal), with benign conditions. Informed consent for gene expression analysis of the tissue samples was received from each patient prior to surgery, and the study was approved by our institutional ethics committee. HCC staging was performed according to the TNM staging system. Adjacent tissue samples were located within 1 cm of the tumor margin and were confirmed to be non-cancerous by pathological examination.

Animal model

A total of 90 male ICR (Imprinting Control Region) mice, purchased and maintained in the animal center of Nanjing Medical University, were randomly divided into nine groups with ten animals per group. Of these, 80 were injected intraperitoneally with 25 mg/kg DEN. The remaining ten mice were injected intraperitoneally with PBS and served as controls. Mice were sacrificed every month following DEN injection and animals were designated according to group based upon the time point of sacrifice. The liver was isolated from each animal and tumor tissue was separated from adjacent tissue. Samples of these tissues were stored in liquid nitrogen for RNA and protein extraction; additional
samples were fixed in 4% paraformaldehyde for histological analysis.

**SOLiD Sequencing**

HCC and corresponding adjacent tissue total RNA samples (1 μg) from ten paired males were processed into sequencing libraries using the Small RNA Expression Kit (SREK, Applied biosystems). Sequencing of small RNAs was generated by SOLiD™ 3 System (performed by Genoson company, Shanghai, China). The sequencing results were aligned with the miRNA precursor sequences of Homo sapiens in the miRBase database (version 14.0, http://www.mirbase.org). The counts for all miRNAs were normalized to the total results for each sample. Detailed information regarding data processing is provided in the supplementary materials and methods.

**Quantitative real-time PCR**

Detailed information is provided in the Supplementary Materials and Methods section.

**Stem loop quantitative real-time PCR**

Detailed information is provided in the Supplementary Materials and Methods section.

**Lentivirus production and transduction**

ERα was excised from a pBabe-puro-ERα plasmid (generously provided by Dr. Luzhe Sun, University of Texas Health Science Center, San Antonio, TX, USA) by restrictive digestion using BamH I and Xho I (New England Biolab, UK) and subcloned into the site of BamH I and Sal I in an HIV type-1 construct, pWPTS-GFP (generously provided by Dr. Trono, University of Geneva, Switzerland), and named pWPT-ERα. Recombinant lentivirus was generated from 293T cells using calcium phosphate precipitation (13). The human HCC cell lines (HepG2 and MHCC-97H) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, CA, USA), was transduced with lentivirus using polybrene (8
μg/ml).

**Cloning of IL-1α promoter and mutagenesis**

The IL-1α promoter was cloned by PCR from genomic DNA of 293T cells. PCR-based cloning was used to generate the deletion construct and point mutation of the 436 bp IL-1α promoter. The amplified products were cloned into the pGL3 luciferase reporter vector (Promega, Madison, WI, USA) using NcoI and XhoI (New England Biolab, UK) sites, respectively, and named pGL3-IL-1α WT, pGL3-IL-1α Mu, and pGL3-IL-1α Del. Plasmid sequencing was performed by Genscript Company (China) with a 3730 DNA analyzer. The sequence of the construct was verified by comparing it to the human genome database.

**Reporter assay**

To conduct the ERα downregulation IL-1α assay, ERα-transduced HepG2 cells were seeded into 24-well plates. The cells were transfected with 2 ng of cytomegalovirus (CMV)-Renilla (Promega, WI, USA), 10 ng of pGL3-IL-1α WT, pGL3-IL-1α Mu, and pGL3-IL-1α Del using Fugene HD (Roche, IN, USA) in accordance with the manufacturer’s instructions. The cells were induced with 100 nM 17β-estradiol (E2) for 24 hours prior to harvesting for the luciferase assay. Firefly luciferase activity was standardized to Renilla luciferase activity as an internal standard of transfection efficiency. All experiments were conducted in triplicate, and the data averaged.

To conduct the ERα downregulation miR-22 assay, HepG2, LM3, and MHCC-97H cells (human HCC cell lines were purchased from the cell bank of the Chinese Science Academy) were seeded into 24-well plates. The cells were transfected with 2 ng of CMV-Renilla (Promega), 10 ng of pGL 3.3’ UTR constructs, and 500 ng of pSuper-Retro-miR-22 or pSuper-Retro-antimiR22 (generously provided by Dr. Didier Picard, University of Genève, Switzerland) or 500 ng pSuper-Retro-control, which contained
the control oligonucleotides, using Fugene HD (Roche) in accordance with the manufacturer’s instructions. The cells were harvested 40 hours post-transfection. Firefly luciferase activity was standardized to Renilla luciferase activity as an internal standard of transfection efficiency. All experiments were conducted in triplicate and the data averaged.

**Hydrogen peroxide induced necrosis**

The human HCC cell lines HepG2 and MHCC-97H, as well as their transduced cell lines, were grown to 80% confluence and treated with E2 at a concentration of 100 nM for 8 hours prior to hydrogen peroxide administration. Cells were treated with 0.5 mM (1×) and 1.0 mM (2×) hydrogen peroxide (14), and cell viability was assessed by Trypan blue staining.

**Western Blot**

Detailed information is provided in the Supplementary Materials and Methods section.

**Immunohistochemistry (IHC)**

All tissues were fixed in 4% paraformaldehyde overnight at 4°C, processed, sectioned into 5 μm slices, and mounted on slides. The sectioned slides were stained for IL-1α (R&D Systems, MN, USA) and ERα (Santa Cruz Biotechnology Inc., CA, USA) using techniques described previously (13). The immunohistochemistry scoring criteria is described in the Supplementary Materials and Methods section.

**Statistical analysis**

The comparison of IL-1α expression between various clinical characteristics was analyzed using the Mann-Whitney U test. The correlation analyses for decreased ERα vs. increased IL-1α expression and IL-1α expression vs. the difference of microRNA expression in HCC and adjacent tissues were performed using linear regression analysis followed by the F-test. Comparisons between two groups
were performed using the Mann-Whitney U-test. Statistical comparisons were conducted using SPSS 13.0 (SPSS Inc. IL, USA). A P-value <0.05 was considered to represent a statistically significant result; to avoid potential error from multiple comparisons, the Bonferroni correction was used.

Results

IL-1α is highly expressed in male tumor adjacent tissue

In order to determine whether the IL-1α-MyD88-IL-6 signaling pathway is activated during HCC development in humans, we examined the respective expression levels using real-time PCR. Tumor tissues and corresponding adjacent tissues from 80 male and 36 female subjects were obtained; tumor adjacent tissues contained abnormal morphology consisting of pseudolobuli, hepatic steatosis, and hepatic hydropsia. Normal tissues from 28 male and 20 female cases of benign liver disease were chosen as male and female controls.

IL-1α, MyD88, and IL-6 were highly expressed in male tumor adjacent tissue compared to control tissue samples (IL-1α, P=0.025; IL-6, P=0.030; MyD88, P=0.021 by Mann-whitney U test, Bonferroni correction was used, Figure 1 A1-A3). Unexpectedly, in male HCC subjects these three proteins exhibited no significant difference compared to controls (IL-1α, P=0.081; IL-6, P=0.064; MyD88, P=0.13 by Mann-whitney U test, Bonferroni correction was used, Figure 1 A1-A3). We also examined IL-1α, MyD88, and IL-6 expression in samples from females, collected over the course of many years. There was no significant difference in IL-1α and IL-6 expression levels in female HCC or adjacent tissue samples compared with controls (Figure 1 A4-A5). However, MyD88 expression was significantly increased in female tumor adjacent tissue samples (P=0.045, Mann-Whitney U test, Bonferroni correction was used, Figure 1 A6), similar to the results obtained in males. Because IL-6 expression has been reported to be regulated by estrogen signaling in mice (11), we also investigated
estrogen signaling in menopausal females. Of 36 female HCC patients, 15 patients were postmenopausal with significantly lower estradiol levels than the remaining 21 premenopausal patients (67.2±20.3 pg/ml versus 207.9±101.5 pg/ml, respectively). In addition, MyD88, IL-1α, and IL-6 expression levels were significantly suppressed in premenopausal patients, most likely due to high estradiol levels (Supplementary Figure S1), indicating that inactivation of estrogen signaling contributed to the upregulation of IL-1α and IL-6. This potentially explains why HCC development is observed in postmenopausal patients. However, we did not find the mechanism of HCC development in premenopausal patients to be estrogen-dependent. This is the first report demonstrating that IL-1α expression is significantly higher in tumor adjacent tissue, and moreover that this expression exhibits a disparity between males and females. We confirmed this finding with IHC and Western blot analysis (Figures 1 B1-B6, Figure 2A).

We also collected demographic data from the HCC patients to investigate the differences between IL-1α expression and HCC characteristics. Our results suggested that IL-1α expression was significantly higher when serum ALT was greater than 45 U/L in both male and female patients, suggesting that IL-1α expression may be related to liver injury (Table 1).

Decreased ERα expression and upregulation of miR-22 occurs in male tumor adjacent tissue in response to increased IL-1α

Based upon the observed sex bias of IL-6 expression in the animal model and previous research regarding the transcriptional regulation of IL-1α (11, 15), we investigated the expression of ERα in male HCC tissue. ERα was dramatically decreased in both tumor and tumor adjacent tissues of males (Figure 2A, 2B1, P=0.00015 and 0.00029, respectively, by Mann-Whitney U test, Bonferroni correction was used). This was confirmed by IHC, which demonstrated almost no ERα expression in male HCC
subjects (Figure 2C1) and weak ERα expression in tumor adjacent tissue (Figure 2 C2). In contrast, high ERα expression was found in both HCC and tumor adjacent tissue in some female patients (Figure 2 A4, C3-C4). We also examined the relationship between increased IL-1α and decreased ERα expression by linear correlation analysis (r=-0.616, P=0.004), and the results indicated a correlation between decreased ERα and increased IL-1α (Figure 3A). We then performed additional experiments to investigate the mechanism of sex disparity of IL-1α expression and its correlation with decreased ERα in HCC subjects. IL-1α secretion was significantly inhibited by E2 in necrotic HCC cell lines that exhibited overexpression of ERα (induced by hydrogen peroxide in vitro, Figure 3B). One ERα binding site was detected in the -1000 bp region of the human IL-1α promoter using the online transcriptional factor prediction tool PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). The luciferase reporter assay indicated that promoter activity was increased when the ERα binding site was mutated in the IL-1α promoter region, suggesting that E2 inhibits IL-1α transcription by binding to its promoter region (Figure 3C).

Previous research on ERα expression in tumor tissue has demonstrated that mutation and methylation of ERα occurs in HCC (16-18); however, few investigations have been conducted to investigate the decreased ERα expression observed in tumor adjacent tissue of the liver. Several studies have demonstrated that microRNAs (miRNAs) such as miR-221, miR-222, and miR-18a downregulate ERα expression by targeting the 3’ UTR in both HCC and breast cancer (19-21). Pandey et al. demonstrated that miR-22 directly represses ERα expression through the 3’ UTR (22). We used SOLiD high-throughput DNA sequencing to screen for related miRNAs in HCC, and the data were then deposited to a public database of Array Express (http://www.ebi.ac.uk/arrayexpress/); accession
number E-MTAB-511. We found that miR-22 was the only microRNA which was not only highly expressed in tumor adjacent tissue but also exhibited a significant linear correlation with IL-1α expression in tumor adjacent tissues (Table 2). Based upon these findings, we conducted stem loop quantitative PCR targeting miR-22, and demonstrated that miR-22 was upregulated in male tumor adjacent tissue compared with HCC tissue and control samples (P=0.014 and P=0.027, respectively, by Mann-Whitney U test, Bonferroni correction was used). In females, we found no upregulation of miR-22 expression (Figure 3E), indicating that differences of miR-22 expression exist between the sexes in human HCC subjects. To elucidate the mechanism, we performed a luciferase reporter assay that demonstrated that miR-22 suppresses ERα transcription by directly targeting its 3′ UTR region in several human HCC cell lines (Figure 3D). In summary, our results clearly demonstrate that IL-1α is highly expressed in human tumor adjacent tissue and that its expression demonstrates a sex bias related to ERα signaling and overexpression of miR-22.

**IL-1α is highly expressed in chronic liver damage and is important for HCC tumorigenesis in mice**

Although there are several published studies focusing on IL-1α, MyD88, and IL-6 expression and signaling during acute liver damage in mice (11, 23), chronic inflammation, a known premalignant stage of HCC, has not been thoroughly investigated. In our study, expression analyses of IL-1α, IL-6, and miR-22 were performed from the 1st to 8th month following DEN administration. There were no visible tumors in the liver until the 4th month following DEN treatment (tumor diameter <0.2 cm; n=6). The volume and number of tumors in the livers of the mice increased over time, and the livers were filled with tumors by the 8th month following DEN administration (tumor diameter >2 cm; n=30). We also investigated expression of IL-1α, IL-6, and miR-22 in chronic hepatitis liver tissue and tumor
adjacent tissue one to six months following DEN injection. We found that IL-1α and miR-22 were also both highly expressed in chronic hepatitis liver tissue and tumor adjacent tissue compared with normal liver tissue (Figure 4 A1 B1, C1, D1-D2). This observation is consistent with the results obtained from human samples in which miR-22 and IL-1α expression levels were both significantly higher in tumor adjacent tissue. Expression levels of miR-22 in tumors of mice exhibit no significant difference compared to normal tissue (Figure 4C2). Both IL-6 and IL-1α are highly expressed during the initial stage of HCC in mice. However, this finding was observed four months post-treatment in tumor tissues (Figure 4A2, B2 and D3) and then the expression levels decreased gradually until six months post-treatment, and expression was almost undetectable at eight months post-treatment (Figure 4 A2, B2, D4-D6).

Discussion

The IL-1α-MyD88-IL-6 signaling pathway is highly expressed in human tumor adjacent tissue, potentially because it is undergoing dysplastic change. Although many studies have previously examined this pathway in HCC, we hypothesized that the associated inflammation and compensatory proliferative response in tumor adjacent tissue may provide further insight into HCC development. We confirmed the findings from previous studies that IL-6 is a multifunctional cytokine and is upregulated in response to hepatic infection (4, 6, 11, 24, 25).

In this study, we demonstrated that IL-1α expression is potentially correlated with human HCC development. A number of previous reports demonstrated that IL-1α acts upstream of IL-6 and that upregulation of IL-1α may be associated with MyD88-mediated IL-6 expression. MyD88 is a key signaling adaptor molecule used by several toll-like receptors (TLRs) that additionally integrates signals generated at the IL-1R, categorized as part of the TLR/IL-1R superfamily (26, 27).
TLR4/MyD88 or IL-1R/MyD88 signaling are necessary for compensatory hepatocellular proliferation following liver injury. Mice deficient in IL-1R or MyD88 display reduced liver injury and inflammatory response, and ultimately develop tumors induced by the hepatocarcinogen DEN (11, 12). Several studies have demonstrated that IL-1α is a ligand to IL-1R, one of the alarmins secreted from necrotic hepatocytes (28, 29). Results of mouse models have indicated that IL-1α secretion from necrotic hepatocytes is essential for stimulation of Kupffer cells, IL-6, and TNF-α; however, the steps involved in this process and during the compensatory proliferation in humans are not fully understood. Our results based on human samples demonstrated that IL-1α is highly expressed in tumor adjacent tissue, particularly in tissues with abnormal morphology. We found that IL-1α expression acts as a general protumorigenic mediator that is released in chronic liver damage, and that it may be a valuable marker for the determination of a premalignant state in humans.

Sex disparity is an area of general interest among HCC researchers. Recently, IL-6 secreted from KCs was proposed as a possible explanation for the sex disparity. Naugler et al. demonstrated that estrogen-mediated inhibition of IL-6 production by KCs reduces HCC risk in females (11). Our findings demonstrated that IL-6 expression is increased in the tumor adjacent tissue of males, not females. Sex disparity in IL-1α expression was also found. In tumor adjacent tissue from males, IL-1α levels were approximately ten fold higher compared with controls; no such difference was found in females. Thus, IL-1α may be responsible for the sex disparity observed in HCC. While the precise mechanisms involved in this process have not yet been elucidated, we hypothesize that sex hormones, which have been previously demonstrated to regulate IL-6 expression, also regulate the expression of IL-1α. Thus, we analyzed ERα expression in HCC tissue obtained from males. We found that ERα is negative in male tumor tissue, suggesting that the protective inflammatory effect mediated by estrogen and ERα is
not present in male HCC tumors. Moreover, we demonstrated a significant linear correlation and
dependence between decreasing ERα expression levels and increasing IL-1α expression levels.
Interestingly, similar results have been reported in breast cancer literature, in which upregulation of
IL-1α also correlates with decreased ERα (30). We conducted a luciferase reporter assay to interpret
the suppression effect on IL-1α expression regulated by E2; this assay confirmed that the ERα binding
site was essential for the IL-1α suppression effect regulated by E2. Moreover, we demonstrated that
IL-1α secretion in vitro can be suppressed by ERα and E2 in HCC cell lines. These results potentially
explain the high IL-1α expression levels in male tumor adjacent tissue. This was not found to be the
case in females because high estrogen levels confer a protective effect by inhibiting the release of IL-6
and IL-1α from KCs.

Our high-throughput DNA sequencing results indicated that only miR-22 was highly expressed in
male human tumor adjacent tissue, and correlated with low ERα and high IL-1α expression. We
confirmed these results through stem-loop PCR. The results demonstrated, for the first time, that
miR-22 was significantly up-regulated in male HCC adjacent tissues and down-regulated in HCC
tissues; these findings were in concordance with previous research concerning the anti-proliferative
role of miR-22 in HCC tissues (31). The results of the luciferase reporter assay indicated that miR-22
could suppress ERα transcription by directly targeting its 3′ UTR region. We outlined our proposed
model of HCC development in human males: E2 and ER repress IL-1α expression in normal
hepatocytes once chronic liver inflammation has occurred, and increasing miR-22 levels result in
downregulation of ERα expression, which in turn stimulates IL-1α transcription. Increased secretion of
IL-1α from necrotic hepatocytes stimulates Kupffer cells, resulting in compensatory proliferation and
tumorigenesis.
Although previous reports have demonstrated that increased IL-1α expression during acute liver inflammation 4-48 hours following DEN injection (28) results in low HCC in IL-1R knockout mice (12), few studies have reported on IL-1α expression during chronic inflammation. It is difficult to fully simulate the role of IL-1α in HCC development; therefore, the results from the dynamic monitoring of IL-1α expression were valuable because IL-1α expression increased significantly in chronic hepatitis liver tissue and tumor adjacent tissue from the 1st to 6th month following DEN administration. It was not feasible to obtain tumor adjacent tissue samples after seven months because the livers of the mice became filled with tumors. However, earlier time-points (e.g., six months) demonstrated very high levels of IL-1α expression in tumor adjacent tissue. IL-1α expression was negligible in tumors with a diameter greater than 1.5 cm, similar to the results obtained in human tissue. Unexpectedly, in the present study we observed that expression of IL-1α increased significantly in tumor tissues (diameter < 0.2 cm) during the 4th month and decreased as the tumor volume increased; at 5-8 months. IL-1α was highly expressed and restricted to tumor cells with larger nuclei compared to the surrounding normal cells. Although the mechanism is unclear, we postulate that high IL-1α expression in sprouting tumor cells potentially activates signaling pathways such as the NF-κB pathway, which exhibits anti-apoptotic and tumor growth properties and has been demonstrated to be activated in HCC (32-34).

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References


19. Adams BD, Furneaux H, and White BA. The micro-ribonucleic acid (miRNA) miR-206 targets the human estrogen receptor-alpha (ERalpha) and represses ERalpha messenger RNA and protein


Figure legends

Figure 1. IL-1α is highly expressed in human male tumor adjacent tissue. Transcriptional expression of IL-1α, IL-6, and MyD88 were detected in tumor tissue, adjacent tissue, and normal tissue. Data represent the value of relative mRNA expression of each parameter compared with GAPDH. A1-A3: Expression of IL-1α, IL-6, and MyD88 in male tumor adjacent tissue (n=80) and male normal tissue (n=28). A4-A6: IL-1α, IL-6 and MyD88 expression in female tumor adjacent tissue (n=36), and female normal tissue (n=20). Data are presented as the mean ± SD. B1, B2: IL-1α expression in male and female liver cancer (×200). B3, B4: IL-1α expression in male and female tumor adjacent cirrhotic tissue; pseudolobuli are present (×100). B5, B6: IL-1α expression in male and female tumor adjacent tissue (×400) with steatosis.

Figure 2. Decreased ERα expression in human male HCC. A: Western blot analyses for ERα and IL-1α expression. Expression in tumor adjacent tissue (A1) and HCC (A2) of ERα and IL-1α from 9 males. Expression in tumor adjacent tissue (A3) and HCC (A4) of ERα and IL-1α from 9 females. A5: Expression of ERα and IL-1α in male normal liver tissue. A6: Expression of ERα and IL-1α in female normal liver tissue. A7: ERα and IL-1α expression relative to GAPDH in normal liver tissue. Data are presented as the mean ± SEM, group 1-6, respectively: male tumor adjacent tissue, male HCC tissue, female tumor adjacent tissue, female HCC tissue, male normal liver tissue, and female normal liver tissue. Experiments were performed in triplicate. B1: Relative expression of ERα, corresponding adjacent tissue (n=80), and male normal tissue (n=28). Data are presented as the mean ± SD. B2: Relative expression of ERα in human female HCC tissue, tumor adjacent tissue (n=36), and female normal tissue (n=20). C1-C2: ERα expression in male tumor and adjacent tissues (×200). C3-C4: ERα expression in female tumor and adjacent tissues (C3, ×200, C4, ×400).
Figure 3. IL-1α expression was regulated by ERα, which correlated with miR-22 upregulation in male tumor adjacent tissue. A: Linear correlation between decreased ERα and increased IL-1α in male tumor adjacent tissue (r=-0.616, P=0.004). Increased IL-1α (ΔIL-1α) were obtained by relative IL-1α expression value in each male adjacent tissue minus the IL-1α mean expression value in the male normal group (ΔIL-1α=IL-1α(male adjacent)x−Mean IL-1α(male normal)). Decreased ERα (ΔERα) were obtained by the relative ERα expression value in each male adjacent tissue multiplied by 100 and then minus the ERα mean value in male normal group (ΔERα=ERα(male adjacent)x×100-MeanERα(male normal)).

B: Western blot detection of IL-1α and ERα expression in hydrogen peroxide treated HCC cell lines, HepG2 and MHCC-97H cells transduced with empty vector, and ERα (“1×” and “2×” represent hydrogen peroxide concentrations 0.5 mM and 1 mM). C: E2 repressed luciferase activity of the wild-type IL-1α promoter (WT), but not those of the target mutation (MU) and target deleted (DEL) IL-1α promoters. The data in the control group were obtained from the pCMV-Renilla fluorescence intensity. D: miR-22 repressed luciferase activity of the wild-type full-length 3′ UTR reporter in HepG2, LM3 and MHCC-97H cell lines. The data in the control group was obtained from the pCMV-Renilla fluorescence intensity. E1: Relative expression of miR-22 in male HCC tissue, tumor adjacent tissue (n=80), and male normal tissue (n=28) normalized by U6; data are presented as the mean ± SD. E2: Relative expression of miR-22 in female HCC tissue, tumor adjacent tissue (n=36), and female normal tissue (n=20) normalized by U6. Data are presented as the mean ± SD.

Figure 4. IL-1α is highly expressed during chronic liver damage. A1: Expression of IL-1α in mice with chronic hepatitis liver tissue and tumor adjacent tissue (n=10) from one to six months following DEN injection. A2: Expression of IL-1α in tumor tissue (n=10) from mice four to eight months following DEN injection. B1: Expression of IL-6 in mice with chronic hepatitis liver tissues and tumor adjacent tissue
(n=10) from one to six months following DEN injection. B2: Expression of IL-6 in tumor tissue (n=10) from mice four to eight months following DEN injection. C1: Expression of miR-22 in mice with chronic hepatitis liver tissues and tumor adjacent tissue (n=10) one to six months following DEN injection. C2: Expression of miR-22 in tumor tissue (n=10) from mice four to eight months following DEN injection. All data are presented as the mean ± SEM. Mann Whitney U test, Bonferroni correction was used. D1-D6: Expression of IL-1α in DEN-injected mice. D1, D2: IL-1α expression in tumor adjacent tissue of mice four months following DEN injection (D1×100 and D2×400). D3: IL-1α expression in tumor tissue from mice four months following DEN injection (×400). D4: IL-1α expression in tumor tissue from mice five months following DEN injection (×400). D5: IL-1α expression in tumor tissue from mice six months following DEN injection (×400). D6: IL-1α expression in tumor tissue from mice eight months following DEN injection (×400).

Abbreviations used in this paper: IL-1α, Interleukin 1α; IL-6, Interleukin 6; DEN, diethylnitrosamine; HCC, heptocellular carcinoma; ERα, estrogen receptor α; HBV, hepatitis B virus; miRNA, microRNA.
Table 1. Clinical Characteristics of 116 HCC patients Grouped by Sex

<table>
<thead>
<tr>
<th>Patient Demographics</th>
<th>Male (Mean±SD)</th>
<th>Female (Mean±SD)</th>
<th>P value(^a) (male, female)</th>
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<tr>
<td>No. of patients</td>
<td>80</td>
<td>36</td>
<td>ND</td>
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<td>post-Menopause</td>
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<td></td>
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<td>Age in years</td>
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<td>0.307±0.513, n=47</td>
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<td></td>
<td>&gt;48</td>
<td>0.327±0.731, n=33</td>
<td></td>
</tr>
<tr>
<td>HbeAg</td>
<td>negative</td>
<td>0.215±0.427, n=18,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>positive</td>
<td>0.652±0.954, n=62,</td>
<td></td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>absent</td>
<td>0.904±1.197, n=8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>present</td>
<td>0.248±0.4767, n=71</td>
<td></td>
</tr>
<tr>
<td>ALT, U/L</td>
<td>≤45</td>
<td>0.183±0.462, n=42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;45</td>
<td>0.503±0.689, n=38</td>
<td></td>
</tr>
<tr>
<td>AFP, ng/ml</td>
<td>≤13.6</td>
<td>0.392±0.596, n=23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;13.6</td>
<td>0.279±0.617, n=57</td>
<td></td>
</tr>
<tr>
<td>Tumor size, cm</td>
<td>≤5</td>
<td>0.287±0.349, n=43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;5</td>
<td>0.492±0.725, n=37</td>
<td></td>
</tr>
<tr>
<td>Tumor multiplicity</td>
<td>solitary</td>
<td>0.386±0.681, n=52</td>
<td></td>
</tr>
<tr>
<td></td>
<td>multiple</td>
<td>0.234±0.524, n=28</td>
<td></td>
</tr>
<tr>
<td>Vascular invasion</td>
<td>absent</td>
<td>0.312±0.664, n=26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>present</td>
<td>0.366±0.637, n=54</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td>I+II</td>
<td>0.426±0.767, n=16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III+IV</td>
<td>0.329±0.621, n=64</td>
<td></td>
</tr>
</tbody>
</table>

ND: not determined

\(^a\) By Mann-Whitney U test, IL-1α expression in male and female adjacent tissues vs. clinicopathological characteristics

\(^b\) P<0.05

\(^c\) P<0.01
Table 2. Differential Expression Pattern of miRNAs in 10 paired male HCC-adjacent tissues

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Count (Mean±SD)</th>
<th>Fold change</th>
<th>P Value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>R²&lt;sup&gt;b&lt;/sup&gt;</th>
<th>P Value&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCC Adjacent</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miRNA Up-regulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-21</td>
<td>13665±2186</td>
<td>4121±224</td>
<td>3.29(2.65-3.92)</td>
<td>&lt;0.0001</td>
<td>0.07131</td>
</tr>
<tr>
<td>miR-224</td>
<td>25238±3011</td>
<td>6958±483</td>
<td>3.77(3.02-4.53)</td>
<td>&lt;0.0001</td>
<td>0.0045</td>
</tr>
<tr>
<td>miR-10b</td>
<td>8302±1454</td>
<td>4111±1001</td>
<td>3.24(1.37-3.01)</td>
<td>&lt;0.0001</td>
<td>0.00027</td>
</tr>
<tr>
<td>miR-221</td>
<td>13608±3672</td>
<td>8184±1180</td>
<td>1.82(1.09-2.55)</td>
<td>0.0016</td>
<td>0.02028</td>
</tr>
<tr>
<td>miR-222</td>
<td>7736±1378</td>
<td>3916±1003</td>
<td>2.27(1.31-3.21)</td>
<td>&lt;0.0001</td>
<td>0.01394</td>
</tr>
<tr>
<td>miR-18</td>
<td>12058±2272</td>
<td>3804±1272</td>
<td>3.79(2.01-5.58)</td>
<td>&lt;0.0001</td>
<td>0.00597</td>
</tr>
<tr>
<td>miRNA Down-regulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-199a</td>
<td>10472±2697</td>
<td>14921±2185</td>
<td>0.67(0.47-0.87)</td>
<td>0.0023</td>
<td>0.00179</td>
</tr>
<tr>
<td>miR-199b</td>
<td>4700±1779</td>
<td>7325±1779</td>
<td>0.60(0.33-0.87)</td>
<td>0.0022</td>
<td>0.08785</td>
</tr>
<tr>
<td>miR-200b</td>
<td>4587±1133</td>
<td>9533±2324</td>
<td>0.49(0.27-0.72)</td>
<td>0.0011</td>
<td>0.0718</td>
</tr>
<tr>
<td>miR-145</td>
<td>28921±3290</td>
<td>62232±9953</td>
<td>0.46(0.35-0.58)</td>
<td>0.0017</td>
<td>0.3343</td>
</tr>
<tr>
<td>miR-22</td>
<td>6234±1615</td>
<td>15689±2801</td>
<td>0.41(0.27-0.60)</td>
<td>&lt;0.0001</td>
<td>0.8127</td>
</tr>
</tbody>
</table>

<sup>a</sup> By paired t-test. Comparison of various microRNA expression in paired human HCC and adjacent tissues. Correction for multiple hypothesis testing was not performed for p-values in the table.

<sup>b</sup> By Linear regression analysis. Correlation between IL-1α level in HCC adjacent tissues and difference of various microRNAs count between male HCC and adjacent tissues.

<sup>c</sup> F test; <sup>d</sup>P<0.01; <sup>e</sup>P<0.0001
miR-22 promotes HBV related hepatocellular carcinoma development in males

Runqiu Jiang, Lei Deng, Liang Zhao, et al.

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