UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE – UFCSPA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Andressa de Freitas Alves

Avaliação de marcadores inflamatórios e do receptor de estrogênio em pacientes com carcinoma hepatocelular

Porto Alegre
2016

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Dissertação submetida ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Mestre.

Orientador: Dra. Márcia Giovenardi

Porto Alegre
2016

Catalogação na Publicação

Alves, Andressa de Freitas
Avaliação de marcadores inflamatórios e do receptor de estrogênio em pacientes com carcinoma hepatocelular / Andressa de Freitas Alves. -- 2017.
63 f. : il., graf., tab. ; 30 cm.

Dissertação (mestrado) -- Universidade Federal de Ciências da Saúde de Porto Alegre, Programa de Pós-Graduação em Ciências da Saúde, 2017.

Orientador(a): Profª Drª Márcia Giovenardi.

1. Carcinoma hepatocelular. 2. Inflamação. 3. Citocinas. 4. Estrogênio. 5. Fígado. I. Título.

Sistema de Geração de Ficha Catalográfica da UFCSPA com os dados fornecidos pelo(a) autor(a).

DEDICATÓRIA

Aos meus pais e irmã, por todo amor e apoio
nessa e em todas as etapas da minha vida.

AGRADECIMENTOS

Nenhum trabalho é feito somente com o esforço de uma pessoa, por isso quero agradecer a cada um dos colaboradores deste trabalho:

Acima de tudo, a Deus.

À minha família, por tudo.

À Dra. Marcia Giovenardi, que, muito mais que uma orientadora, me ouviu, me guiou, me ensinou e me auxiliou nesse Mestrado e nos últimos anos.

À Dra. Vanessa Baldissera por ter me introduzido a essa pesquisa, me ensinado e me apoiado durante os anos, além da amizade.

Aos professores colaboradores desse projeto:

Dr. Thadeu Czerski e Dr. Paulo Fontes, pelo apoio à pesquisa, seleção dos pacientes e coleta das amostras.

Dra. Marilene Porawski, pelas conversas e ajuda e resolução de dúvidas, além do apoio para a execução do projeto.

Dra. Marilda Fernandes, pelos ensinamentos, auxílio e resolução de dúvidas para a contagem e quantificação em imuno-histoquímica.

Dr. Eduardo Chiela, por ter me recebido e ter sido muito prestativo na resolução de dúvidas e elaboração de ideias.

Dr. Alberto Rasia Filho, por ter me auxiliado na captação das imagens, sempre sendo muito simpático e prestativo comigo.

Dra. Silvana de Almeida, pelas conversas e auxílio na estatística do trabalho, além da amizade.

Além disso, também às técnicas de laboratório, sempre solícitas, que me ensinaram e me ajudaram, em especial à Rosalva Meurer, Teresinha Stein, Keli Reiter e as meninas do Laboratório de Patologia.

Por fim, a UFCSPA e a todos os professores, familiares e amigos que, de alguma forma, me auxiliaram na chegada até aqui.

Muito obrigada!

RESUMO

Introdução: O carcinoma hepatocelular (CHC) é um tipo de câncer relacionado à inflamação, já que 90% dos casos se desenvolvem a partir de um estado de inflamação crônica. A inflamação, quando em excesso, pode afetar a homeostase tecidual. Citocinas e mediadores inflamatórios são componentes imunológicos capazes de influenciar o funcionamento de células e tecidos. Além disso, o receptor de estrogênio (ER) parece ter um papel importante na hepatocarcinogênese. **Objetivo:** Avaliar a expressão de marcadores inflamatórios e o ER em pacientes com carcinoma hepatocelular. **Metodologia:** Foram analisados os dados de 143 pacientes com e sem carcinoma hepatocelular, provenientes da ISCMPA. Foi realizada a imuno-histoquímica para a COX-2, NF-κB, TNF-α e ER no tecido hepático parafinizado dos pacientes. Quatro campos representativos foram capturados em 200x e avaliou-se o percentual de área corada, o valor de intensidade de marcação e a contagem de núcleos positivos para ER com o software ImageJ 1.50. **Resultados:** O sexo masculino foi o mais prevalente em ambos os grupos, com média de idade em torno da quinta década de vida. As etiologias mais frequentemente encontradas foram a cirrose e hepatite C. Foi encontrada diferença significativa entre os grupos para o percentual de área marcada (p=0,04) para a COX-2 e para a intensidade de marcação para o TNF-α (p=0,03). Não foi encontrada diferença significativa em nenhum dos outros parâmetros e marcadores avaliados. **Conclusão:** A COX-2 e TNF-α foram os marcadores que apresentaram diferença significativa entre os grupos estudados, configurando-os como marcadores elegíveis de serem estudados de forma mais aprofundada em relação ao perfil imuno-histoquímico e do papel deles no desenvolvimento do CHC .

Palavras-chaves: Carcinoma hepatocelular. Inflamação. Citocinas. Ciclooxigenase-2. Fator de necrose tumoral alfa. Fator nuclear kappa B. Receptor de estrogênio.

ABSTRACT

Introduction: Hepatocellular carcinoma (HCC) is a type of cancer related to inflammation, since 90% of cases are developed in a chronic inflammation condition. Inflammation, when in excess, can affect tissue homeostasis. Cytokines and inflammatory mediators are immunological components that can influence the functioning of cells and tissues. In addition, estrogen receptor (ER) appears to play an important role in hepatocarcinogenesis. **Objective:** To evaluate expression of inflammatory markers and ER in patients with hepatocellular carcinoma. **Methodology:** Data from 143 patients from ISCMPA, with and without hepatocellular carcinoma, were analyzed. Immunohistochemistry was performed for COX-2, NF-κB, TNF-α and ER in paraffin-embedded hepatic tissue. Four representative fields were captured at 200x magnification and percentage of stained area, intensity of staining and counting of ER positive nucleus were evaluated with ImageJ 1.50 software. **Results:** Males were most prevalent in both groups, with mean age around fifth decade of life. The most frequent etiologies found were cirrhosis and hepatitis C. There was a significant difference between groups for percentage of marked area ($p = 0.04$) for COX-2 and for intensity of staining for TNF-α ($p = 0.03$). No significant difference was observed in any of other parameters evaluated. **Conclusion:** We found a significant difference between groups for COX-2 and TNF-α, which configure them as eligible markers for further studies about their immunohistochemistry profile and their role in HCC development.

Key-words: Hepatocellular carcinoma. Inflammation. Cytokines. Ciclooxinenase-2. Tumoral necrosis factor alpha. Nuclear kappa B factor. Estrogen receptor.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS

AA	Ácido araquidônico
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CHC	Carcinoma hepatocelular
COX-1	Ciclooxigenase 1
COX-2	Ciclooxigenase 2
EE	*Ethinyl estradiol*
ER	Receptor de estrogênio/estrogen receptor
HBV	Hepatitis B virus
HBx	Proteína x do vírus da hepatite B
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
IKK	I kappa B quinase
mPGES-1	Prostaglandina E sintase microssomal 1
NF-kB	Fator nuclear kappa B
NO	Óxido nítrico
PGE2	Prostaglandina E2
PGH2	Prostaglandina H2
PGs	Prostaglandinas
ROS	Espécies reativas de oxigênio
TNFR-1	Receptor do fator necrose tumoral 1
TNFR-2	Receptor do fator necrose tumoral 2
TNF-α	Fator de necrose tumoral alfa
VHB	Vírus da hepatite B
VHC	Vírus da hepatite C

SUMÁRIO

1. INTRODUÇÃO

1.1. EPIDEMIOLOGIA DO CARCINOMA HEPATOCELULAR

O carcinoma hepatocelular (CHC) é o principal tipo de tumor primário dos hepatócitos, constituindo de 70-80% dos tumores primários do fígado[1,2,3,4]. É considerado o sexto tipo mais comum de câncer em homens e o sétimo em mulheres, e a segunda causa de mortalidade por câncer mundialmente[1]. Segundo dados de 2008, sua incidência global apresentou-se em cerca de 750.000 novos casos com uma mortalidade semelhante de 695.900 pessoas [2,5,6], porém variando geograficamente. As maiores taxas encontram-se na Ásia e na África, sendo que 75% dos casos ocorrem no continente asiático[1]. No Brasil, o CHC apresenta-se como a oitava causa de morte por câncer no país[7].

Os fatores de risco para o CHC são muito bem definidos, mas qualquer condição que leve a condição de hepatopatia crônica e, eventualmente, uma cirrose pode tornar-se um potencial agente oncogênico[4,7]. Desse modo, condições como a hepatopatia crônica e a cirrose, principalmente relacionada ao vírus da hepatite B (VHB), vírus da hepatite C (VHC), ao álcool e à aflotoxina B1, apresentam-se como os agentes mais relevantes, sendo responsáveis por até 90% dos casos de CHC. Embora outras causas também estejam relacionadas em menor frequência com CHC, elas acabam representando um menor impacto global[1,3,5].

Uma grande variação das taxas de incidência do CHC é vista entre diferentes populações que vivem numa mesma região. Nos Estados Unidos, por exemplo, encontra-se uma taxa duas vezes maior em asiáticos do que em afro-americanos que, por sua vez, são duas vezes maiores do que em caucasianos. Uma possível explicação para isso seriam as diferenças na prevalência e tempo de aquisição de fatores de risco para o CHC entre as etnias[4,5].

Estudos epidemiológicos indicam que a incidência de CHC é maior em homens do que em mulheres, com uma razão homem/mulher entre 2:1 e 4:1, sendo que as doenças crônicas hepáticas progridem mais rapidamente para cirrose nos homens[8]. Dentre as possíveis razões para essa disparidade está na diferença da exposição aos fatores de risco, mas também é estudado o possível efeito na diferença de hormônios sexuais[4].

1.2. FATORES DE RISCO PARA O CHC

1.2.1. Cirrose

A cirrose está presente em cerca de 80 a 90% dos casos de CHC, muitas vezes acompanhada de fibrose moderada a avançada. As causas para a cirrose podem ser diversas, mas destacam-se as causadas pelos vírus da hepatite que abrangem 80% dos casos de cirrose mundialmente[5]. A cirrose é resultado de proliferação celular e aumento de síntese de DNA em nódulos em regeneração cujos processos podem desencadear modificações capazes de alterar funcionalmente proteínas regulatórias[7]. Além de por si ser uma condição pré-maligna, a cirrose promove uma susceptibilidade ao fígado perante outros tipos de agente oncogênicos externos, uma vez que células estão mais susceptíveis a alterações no DNA[9].

1.2.2. Vírus da Hepatite B

A infecção por VHB é considerada um dos principais fatores de risco para o CHC, uma vez que abrange mais de 50% dos casos mundialmente[1,5,6], tendo principal destaque em áreas de altas taxas de incidência como África Subsaariana e Leste Asiático[1,10]. Nessas áreas, o principal método de transmissão é via vertical enquanto que em áreas de baixas incidências como Noroeste da Europa, Oceania, América do Sul e do norte a via sexual (horizontal) é a mais comum[1,4,5]. Estima-se que a integração do VHB no DNA do hospedeiro induza ao dano, levando a um risco aumentado de CHC e que o conteúdo de carga viral e a duração da infecção contribuam para um risco acumulado de efeito prolongado[6].

1.2.3. Vírus da Hepatite C

O VHC, por sua vez, também se apresenta como um fator de risco importante para o CHC, estando presente em torno de 30% dos casos[5], sendo a causa mais comum em países desenvolvidos como Estados Unidos, países da Europa e Japão[1,2,5]. A principal via de contaminação pelo vírus quando relacionado ao CHC se dá por transmissão via sangue contaminado[4,6]. Contrariamente ao VHB, o mecanismo de desenvolvimento tumoral pelo VHC ainda é desconhecido, mas conclui-se que o vírus desempenhe um papel importante no processo uma vez que

se observa um risco duas vezes maior do desenvolvimento de CHC em pacientes que falham na erradicação do VHC com a terapia antirretroviral[5].

1.2.4. Álcool e outras causas

O álcool é considerado um fator de risco devido, ao que parece, somente ao fato de levar a uma doença alcoólica do fígado, em particular a cirrose. Estudos sugerem que a maior contribuição do álcool para o CHC seria o de acelerar a progressão da fibrose em pacientes com hepatite B e C crônicas, ou seja, o efeito sinérgico de larga ingestão de álcool e a infecção pelo VHC ou VHB[2,4,5]. Outra substância também apontada como um fator de risco é a aflotoxina B, uma micotoxina derivada do fungo *Aspergillus* sp, cujo papel relacionado ao CHC se deve ao fato de possuir um potente efeito hepatocarcinogênico[2,4].

1.3. PROGNÓSTICO DO CHC

O CHC apresenta-se como um tipo de malignidade com limitadas opções de tratamento. Dentre as alternativas, com intenção curativa, disponíveis estão a radiofrequência, a ressecção cirúrgica e o transplante de fígado[6,11]. No entanto, somente 35% destes pacientes são candidatos aos tratamentos citados acima, sendo possível, no restante dos casos, apenas tratamentos paliativos, como a quimioembolização e, mais recentemente, o tratamento com o Sorafenib® [6,12]. O conhecimento de indicadores prognósticos é de grande utilidade para uma orientação adequada na escolha do tratamento e na seleção dos pacientes para as terapêuticas propostas.

1.4. INFLAMAÇÃO E CÂNCER

A inflamação constitui-se como uma reação complexa em resposta à infecção, à exposição de toxinas ou à lesão celular e que possui envolvimento de proteínas plasmáticas e células do sistema imune[13]. De um modo geral, a função primária da inflamação consiste em rapidamente destruir ou atenuar qualquer perturbação da homeostasia[14]. No entanto, quando o processo de inflamação está desregulado, afetando cronicamente a homeostase de um tecido, ela pode levar a distúrbios nas interações entre os sistemas imune inato e adaptativo causando, por sua vez, desordens inflamatórias crônicas[14,15].

Dessa forma, a inflamação quando crônica, não resolvida, persistente e desregulada pode provocar dano, necrose e, até mesmo, transformação maligna das células do órgão afetado[14]. A falha na ativação e inativação do sistema imune diante desse excesso de inflamação pode provocar remodelação tecidual excessiva, perda da arquitetura tecidual devido ao crescimento excessivo da célula, apoptose ou necrose e alterações em proteínas relacionadas com estresse oxidativo e dano (Figura 1). Portanto, esse conjunto de fatores pode levar a um aumento no risco de desenvolvimento de câncer[15].

Figura 1. Condições inflamatórias levando a iniciação tumoral.

Fonte: Figura adaptada de Raposo et al.[16]

Estudos sugerem que a inflamação pode ter um papel importante em promover condições que levem ao câncer, seja nas fases de iniciação, promoção, invasão, disseminação metastática e escape do sistema imune[17]. Diversos processos causados pela inflamação excessiva e desregulada levantam essa hipótese, como:

1. A inflamação ser capaz de provocar estresse celular, dano ao DNA e instabilidade genômica[17];

2. Poder levar a alterações na expressão de genes relacionados a cânceres e também alterar proteínas relacionadas ao ciclo celular, reparo de DNA e apoptose após sua tradução[14];

3. Poder levar a formação de espécies reativas de oxigênios (ROS) que também são capazes de desestabilizar o genoma[16];

4. Promover o recrutamento excessivo de células imunes e infiltração das mesmas no tecido, levando também a secreção de fatores de crescimento, citocinas e quimiocinas que podem ter efeitos pró-angiogênicos, de estímulo de crescimento celular, de diferenciação celular e de evasão do sistema imune[16,17,18].

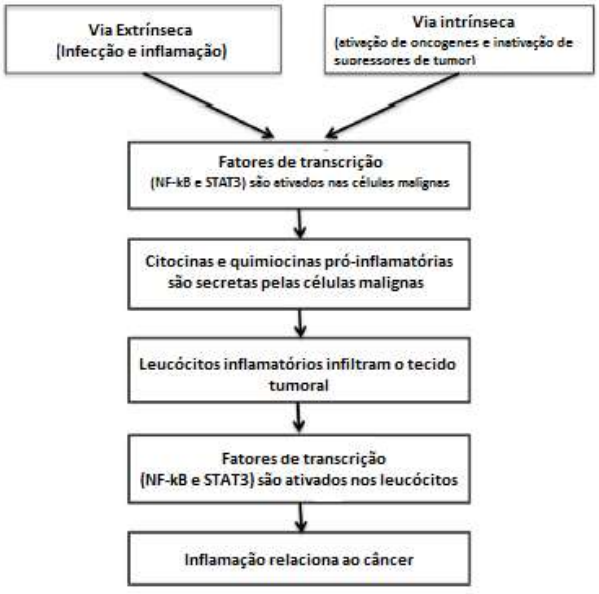
A relação entre a inflamação e o câncer parece abranger duas vias, uma intrínseca e outra extrínseca (Figura 2):

1. Via intrínseca: abrange uma mutação e ativação de oncogenes que regulam o ambiente inflamatório e a inativação de supressores de tumores[16,17];

2. Via extrínseca: abrange condições do ambiente inflamatório que promovem o desenvolvimento do câncer infecção e outros promotores inflamatórios[16,17].

No final, ambas as vias culminam na ação de fatores de transcrição, citocinas e quimiocinas que recrutam os leucócitos para o ambiente tecidual[16].

Figura 2. Vias da inflamação no câncer: intrínseca e extrínseca.



Fonte: Traduzida e adaptada da imagem original de Maiorov et al. [17]

Cerca de 20% dos tipos de cânceres são associados à inflamação crônica cujas causas estão relacionadas a infecções, exposição a agentes químicos e doenças autoimunes[19]. Alguns exemplos de iniciação tumoral relacionada à inflamação como contribuinte são: agentes infecciosos promovendo um ambiente inflamatório como a *Helicobacter pylori* em carcinoma gástrico e os vírus da hepatite em CHC; doenças autoimunes como a doença inflamatória intestinal no câncer colorretal; inflamação subclínica devido a obesidade em cânceres de fígado e agentes ambientais carcinogênicos como o cigarro para o câncer de pulmão[16,19].

1.5. INFLAMAÇÃO E CHC

O CHC configura-se como um tipo de câncer relacionado à inflamação, uma vez que, cerca de 90% dos casos se desenvolvem a partir de um estado de inflamação crônica[20,21,22]. Juntamente a isso, os principais fatores de risco para o desenvolvimento do CHC que são a infecção pelos vírus da hepatite, cirrose e o consumo excessivo de álcool, estão relacionados com o desenvolvimento de inflamação crônica tecidual[22]. Alguns estudos em animais já demonstraram que processos inflamatórios crônicos intra-hepáticos são capazes promover a hepatocarcinogênese[21].

O fígado é um órgão intimamente associado ao sistema imune. Este órgão é repleto de células relacionadas com a imunidade e defesa do organismo, tais como: células dendríticas, células de *Kupffer*, células mielóides do fígado, células endoteliais sinusoidais, além de linfócitos[15,23]. Dessa forma, um dano celular nos hepatócitos pode levar ao recrutamento e ação desses componentes levando a respostas celulares que, se prolongadas, podem promover a proliferação e morte celular[15]. Por sua vez, a necrose dos hepatócitos pode levar a um aumento da mutagênese celular e, com acumulação gradual das mesmas, pode causar mudanças genéticas na célula levando a transformação maligna [3,15].

Uma das principais hipóteses para a relação entre a inflamação crônica e o CHC está relacionada com o papel o qual o ambiente tecidual desempenha na formação do tumor[3,24]. Quando as células presentes no fígado e o sistema imune são recrutados por um estímulo ou dano, estas são capazes de secretar fatores de

crescimento, citocinas, quimiocinas, radicais livres e outras substâncias que podem contribuir para a iniciação e progressão tumoral[3,14].

Um dos componentes do sistema imune diretamente relacionado com a inflamação e sua progressão, bem como, sendo capaz de influenciar o funcionamento das células são as citocinas[14,15]. As citocinas são proteínas extracelulares e hidrossolúveis produzidas por diversos tipos celulares que medeiam reações inflamatórias e imunes, funcionando como um meio de comunicação entre as células imunológicas[13,25]. A principal função das citocinas é a de conduzir a resposta inflamatória até o local da lesão ou infecção com o objetivo de promover a resolução[25]. Além desta função principal, elas são capazes de regular o crescimento, diferenciação e ativação das células do sistema imune. Dessa forma, quando falham em resolver o dano no tecido, promovem uma infiltração excessiva de células imunes. Isso, por sua vez, pode impactar em diversos estágios de formação e progressão do câncer[15].

As citocinas são divididas em duas classes: as pró-inflamatórias (Interleucinas 1,2,6,7; fator de necrose tumoral alfa e interferon gama) e as anti-inflamatórias (Interleucinas 4,5,8,10)[13,15,25].

1.5.1 Fator de necrose tumoral alfa e Fator nuclear kappa B

O fator de necrose tumoral alfa (TNF-α) é produzido por monócitos, macrófagos e linfócitos T[13,25]. O TNF- α é um dos mediadores mais precocemente produzidos na resposta inflamatória e promove vários efeitos sobre o endotélio e os leucócitos[13,25]. Essa citocina foi primeiramente associada a ações anticancerígenas, mas atualmente já se sabe do seu papel pró-tumorigênico, estando envolvido em diversos processos como[18,26]:

1. A criação de um ambiente tumoral que estimula o crescimento e sobrevivência das células malignas através da indução do fator nuclear kappa B (NF-κB)[14];

2. A estimulação da infiltração de células inflamatórias no tecido[14];

3. Promoção da angiogênese, invasão celular, migração e supressão de linfócitos citotóxicos e macrófagos ativados[14,26];

4. Contribuição para a iniciação de tumores através da estimulação da produção de ROS e óxido nítrico (NO) que podem causar mutações no DNA[14].

Além disso, o TNF-α pode atuar ativando uma cascata de sinalização através da ativação de IKK (complexo enzimático de quinases) que resulta na ativação e translocação do NF-κB ao núcleo e induz genes-alvo capazes de aumentar a proliferação e sobrevivência celular[27]. Também pode induzir a necrose e apoptose através de receptores de TNF do tipo I (TNFR-1) e tipo II (TNFR-2)[28].

Em modelo animal DEN-induzido já foi demonstrado que o TNF-α é capaz de favorecer o desenvolvimento de CHC ao promover a hepatoesteatose[20]. Níveis aumentados de TNF-α foram relatos em pacientes com câncer de tonsila[29], câncer colorretal[30], câncer de próstata[31] e em fígados cirróticos[32]. Além disso, a supressão de TNF-α *in vitro* foi capaz de suprimir a proliferação e invasão celular em linhagem de células de câncer de vesícula biliar[33] e diminuir as metástases de câncer colorretal para o fígado[34]. Também foi encontrada uma relação do TNF-α com os mecanismos de resposta imune inata contra o HBV e com as concentrações da proteína viral HBx [35,36].

O principal mediador intracelular do TNF-α é o NF-κB cujos genes alvos estão envolvidos na proliferação e sobrevivência celular[20]. O NF-κB é composto por uma família de fatores de transcrição composta que normalmente ficam retidos no citoplasma, translocando-se ao núcleo quando ativados[13,20,22]. O NF-κB é responsável por diversas funções como a regulação da expressão de muitas citocinas e moléculas de adesão. Além disso, também coordena vias centrais de sinalização na ativação de respostas imunes inatas e adaptativas[14] e induz a expressão de enzimas chave na inflamação como a via da síntese de prostaglandina, síntese de NO e fatores angiogênicos[37].

Pelo fato de ser uma peça chave na regulação da imunidade inata e inflamação, evidências recentes sugerem que o NF-κB representa uma ligação molecular entre inflamação e o câncer[20]. Dentre algumas evidências, temos[16,19]:

1. Expressão constitutiva de NF-κB foi identificada em muitos tipos de canceres, dando suporte do seu papel no fenótipo.

2. Desregulação na via do NF-κB já foi documentado extensivamente em malignidade hematológicas e linfomas; Essas apresentaram ativação aberrante do NF-κB que promoveu sobrevivência as células tumorais, protegeu contra a apoptose e favoreceu a oncogênese.

3. A ativação constitutiva de NF-κB é uma marca de doenças inflamatórias crônicas que, por sua vez, são associadas com o aumento da incidência de câncer.

4. A regulação das proteínas anti-apoptóticas Bcl-2 como um resultado da ativação persistente da via NF-κB, com subsequente desregulação da apoptose, foi estabelecida como uma grande causa de resistência a quimioterapia e é associada a uma agressividade clinica maior e pouco sobrevivência em malignidade hematológicas.

Em estudos realizados com animais, já foi visto que a inibição do IκB induziu a morte de hepáticos devido ao acúmulo de ROS[20,22]. Outro estudo demonstrou que a mesma inibição levou ao desenvolvimento espontâneo de CHC em camundongos[3].

O NF-κB parece ter papel duplo no desenvolvimento do CHC. Em estágios iniciais de desenvolvimento do tumor, o efeito anti-apoptótico parece ser desejável porque previne a morte do hepatócito. No entanto, em estágios mais avançados, o NF-κB acaba auxiliando a sobrevivência da célula tumoral. Em células que não constituem o parênquima, a ativação do NF-κB leva a ativação persistente da cascata da inflamação [20,38].

1.5.2. Enzima ciclooxigenase-2

Outro mediador que também tem ganhado destaque nessa relação entre inflamação e câncer é a enzima ciclooxigenase-2 (COX-2). As enzimas da família COX são responsáveis por converter o ácido araquidônico (AA) livre em prostanóides, incluindo prostaglandinas (PGs) e tromboxanos[14,18]. Existem duas enzimas principais: a COX-1 e a COX-2, sendo a COX-2 a mais relacionada com o desenvolvimento e a progressão do câncer. Sua expressão é praticamente negativa

nos tecidos normais e órgãos em situações fisiológicas, exceto pela expressão constitutiva nos rins e cérebro[39].

Porém, a síntese desta enzima será induzida por estímulos mitogênicos e inflamatórios[18,40]. Níveis elevados de COX-2 já foram relatados em tumores como de: mama, próstata, pâncreas, pele, pulmão, bexiga, esôfago, cabeça e pescoço[19,41]. No CHC, já foi reportado que a expressão de COX-2 está relacionada com a presença de células inflamatórias, incluindo macrófagos[20].

A COX-2 está relacionada com a formação de carcinógenos, com a promoção tumoral, inibição da apoptose, angiogênese e processos metastáticos[19,39,40]. A indução de COX-2 ativa a Prostaglandina e sintase microssomal (mPGES-1), uma enzima induzível que catalisa o derivado intermediário da COX-2, a Prostaglandina H2 (PGH2), em Prostaglandina E2 (PGE2), um mediador biológico com efeitos tumorigênicos. O PGE2 é a prostaglandina mais abundante em tumores sólidos e foi demonstrado que influencia o crescimento, migração e invasividade das células turmorais[18]. Pode ainda estimular o crescimento de vasos sanguíneos, inibir a função imune local e regular diversas vias de transdução de sinal que influenciam a proliferação e crescimento de tumores. Além de mediar a ação da citocina TNF-α via NF-κB [39].

Ademais, a COX-2 também pode inibir a apoptose em células cancerosas ao induzir mutação no gene p53 e pode diminuir a atividade das e-caderinas. Essas proteínas possuem a função de inibir a separação das células cancerosas do tecido de origem, aumentando, assim, a invasividade e a chance de metástase[39].

Em estudos realizados no modelo animal, foi demonstrado que a supressão de COX-2 previne o aparecimento de neoplasias em diversos modelos de câncer[18]. Além disso, em linhagem de células de hepatoma, a inibição da enzima pode induzir a supressão do crescimento celular via indução da apoptose[42]. Cheng et al.[43] demonstraram a co-expressão entre a HBx (proteína viral do HBV) e a COX-2 em doenças crônicas hepáticas, levantando a hipótese de que a presença da proteína viral possa induzir a produção da enzima no câncer hepático.

1.5.3.Receptor de estrogênio

Os estrogênios influenciam diversas situações fisiológicas e fisiopatológicas nos mamíferos, como reprodução, sistema cardiovascular, integridade óssea, sistema hepático, cognição e comportamento[44,45]. O fígado, sendo um órgão hormônio-sensível, pode sofrer influência dos diversos hormônios presentes no organismo humano, dentre eles, os hormônios sexuais, como o estrogênio[46].

O efeito dos hormônios sexuais na função e morfologia do fígado já foi destacado em vários estudos [8,44], tendo-se em vista a hipótese de que os mesmos possuem um papel na patogênese da neoplasia hepática[47]. Em modelos animais já foi relatado que o estrogênio apresenta o papel de promotor de tumores, podendo induzir a hepatocarcinogênese. Uma relação entre a expressão de receptores de estrogênios (ERs) e a formação de tumor induzida por estrogênio já foi demonstrada em camundongos transgênicos MT-mESR[48]. Outro estudo demonstrou que 8% de ratas fêmeas que receberam tratamento de *ethinyl estradiol* (EE) por doze meses desenvolveram CHC, revelando, portanto, que o EE causa mutação nos hepatócitos o que leva a formação de adutos de DNA e induz o desenvolvimento do câncer nas células afetadas[8].

Os ERs pertencem a uma família de receptores nucleares que podem regular a expressão de diversos genes[8]. Atua como um mediador na via de transdução de sinal, sendo expresso em uma grande variedade de tipos celulares, além de ter um papel vital na resposta de ação do estrogênio[49]. Já se tem conhecimento de que é responsável por induzir proliferação celular, gerenciar o crescimento celular, programar a morte celular e acumular mutações genéticas durante a divisão celular através da ligação com tanto hormônios endógenos quanto exógenos no câncer de mama[50].

Na inflamação, o papel do estrogênio já foi visto como anti-inflamatório em algumas doenças, inclusive em doenças hepáticas. Seus efeitos estariam relacionados com a inibição da secreção de algumas citocinas pró-inflamatórias, dentre elas o TNF-α[51]. Por outro lado, uma ativação da via NF-κB em tumores positivos para receptor de estrogênio já foi associada com um fenótipo mais agressivo do câncer de mama assim como uma resistência à terapia endócrina[52].

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3. OBJETIVOS

3.1. Objetivo Geral

Avaliar a expressão de marcadores inflamatórios e do receptor de estrogênio em pacientes com carcinoma hepatocelular.

3.2. Objetivos Específicos

- Analisar e comparar a expressão do TNF- α, COX-2 e NF-κB pela técnica de imuno-histoquímica em amostras de espécimes hepáticos de pacientes com e sem carcinoma hepatocelular.

- Analisar e comparar a expressão imuno-histoquímica do receptor de estrogênio em amostras de espécimes hepáticos em pacientes com e sem carcinoma hepatocelular.

- Descrever parâmetros como sexo, idade e fatores etiológicos na amostra estudada.

4. ARTIGO

Name of Journal: World Journal of Gastroenterology
Manuscript Type: Case Control Study

Evaluation of inflammatory markers and estrogen receptor in patients with hepatocellular carcinoma

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ABSTRACT

AIM: To evaluate expression of inflammatory markers and estrogen receptor in patients with hepatocellular carcinoma.

METHODS: Data from 143 patients from ISCMPA, with and without hepatocellular carcinoma, were analyzed. Immunohistochemistry was performed for COX-2, NF-κB, TNF-α and ER markers in paraffin-embedded hepatic tissue previously collected. Four representative fields were captured at 200x magnification and percentage of stained area, intensity of staining and counting of ER positive nucleus were evaluated with ImageJ 1.50 software. The following tests were used in statistical analysis: Chi-Square test for categorical values; Student's t test for COX-2 and NF-κB expression; Mann-Whitney U test for TNF-α and NF-κB (intensity parameter) expression. Significance level was considered as $p<0.05$.

RESULTS: Male sex was most prevalent in both groups of patients of sample, with a mean age around fifth decade of life. The most frequent etiologies found in sample for both groups were cirrhosis and infection of hepatitis C virus, followed by alcohol consumption and infection of hepatitis B virus. It was found a significant difference between case and control groups for percentage of marked area (30,62 ± 9,72 *vs* 24,39 ± 11,77, p = 0.04) for COX-2 marker and for intensity of staining for TNF-α marker (9,19 ± 8,19 *vs* 15,76 ±12,36, p=0.03). No significant difference was found in any of the other parameters evaluated and for NF-kB and ER markers.

CONCLUSION: We found a significant difference between groups for COX-2 and TNF-α, which configure them as eligible markers for further studies about their immunohistochemistry profile and their role in HCC development.

Key words: Hepatocellular carcinoma; Inflammation; Cytokines. Ciclooxinenase-2; Tumor necrosis factor alpha; Nuclear kappa B factor; Estrogen receptor.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary type of hepatic tumor, representing 70-80 % of primary liver tumors[1,2,3,4]. It is considered as the sixth most common cancer worldwide, and as second leading cause of cancer mortality in world[1]. Global incidence was presented as 750,000 new cases with a similar mortality of 695,900 people, according to data from 2008[2,5,6], but varying geographically. Conditions such as chronic hepatitis and cirrhosis, particularly related to hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol and aflatoxin B1 are presented as main risk factors for this cancer[5,3,1].

It is suggested in studies that inflammation may have an important role in promoting conditions that lead to cancer as in stages of initiation, promotion, invasion, metastatic dissemination and evasion of immune system[7]. Cytokines and inflammation mediators are directly involved with inflammation and its progression, having the ability to influence cells and tissues[8,9]. Their main goal is to conduct inflammation response in order to promote resolution of damage[11]. However, when they fail to resolve the problem, their actions can promote excessive immune response that can, in turn, impact in various stages of formation and progression of cancer[8]. Taking this into account, HCC is characterized as a type of cancer associated with inflammation, since about 90% of cases are developed from a state of chronic inflammation[11,12,13].

Tumor necrosis factor alpha (TNF-α) is one of the mediators that are first produced in the inflammatory response. It promotes different effects on endothelium and leukocytes, such as stimulation of adhesion factors expression and stimulation of macrophages and endothelial cell production of chemokines[14,10]. Primarily, TNF-α was associated with anti-cancer actions, but now it is known of its pro-tumorigenic role, being involved in angiogenesis, inflammation, in formation of metastases and activation of tumor survival pathways[15,16]. Increased levels of TNF-α have been reported in patients with tonsil cancer[17], colorectal cancer[18], prostate cancer[19] and cirrhotic livers[20], among others.

The major intracellular mediator of TNF-α are target genes of nuclear factor kappa B (NF-κB) that are involved in cell proliferation and survival[11]. NF-κB is responsible for various functions such as expression regulation of many cytokines and adhesion molecules that are critical elements involved in regulation of immune responses[9]. Previous study[21] suggests that NF-κB appears to have a dual role in development of HCC. In the early stages of tumor growth, its anti-apoptotic effect seems to be desirable because it prevents hepatocytes' death. However, in more advanced stages, NF-κB leads to survival of tumor cells. In cells that are not liver parenchyma, activation of NF-κB leads to persistent activation of the inflammation cascade[21].

Another mediator that has also gained prominence in relationship between inflammation and cancer is cyclooxygenase-2 enzyme (COX-2). COX family of enzymes are responsible for converting arachidonic acid (AA) in free prostanoids, including prostaglandins (PGs) and thromboxane[9,16]. COX-2 is related to formation of carcinogens, tumor promotion, inhibition of apoptosis, angiogenesis, and metastatic processes [22,23,24]. Moreover, elevated levels of COX-2 have been reported in some tumors as: breast, prostate, pancreas, skin, lung, bladder, esophagus, head and neck tumors[24,25]. In HCC, it has been reported that COX-2 expression is related to the presence of inflammatory cells, including macrophages[11] as well as some studies report its relevance in tumor differentiation and relation with HBV infection[26,27,28,29].

Estrogen receptors (ER) also seem to have an important role in liver carcinogenesis. They belong to a family of nuclear receptors involved in regulation of a variety of genes[30], whose actions are induction of cell proliferation, management of cell growth and cell death programming[31]. In animal models, it has been reported that estrogen can promote tumor growth and induce hepatocarcinogenesis[32]. In turn, its role in inflammation seems to be related with anti-inflammatory effects by inhibition of pro-inflammatory citokynes[33]. Although, it was reported that NF-κB activation in ER positive tumors have been associated to a more aggressive phenotype of breast cancer[34].

Considering the role of inflammation in development of cancer, mediators such as TNF-α, NF-kB, COX-2 and ER may have a part in it. Some studies have already investigated the relation of those markers[35,36,37] in development of chronic liver diseases, but not necessarily with HCC human tissue. Therefore, the purpose of our study was to investigate association of cited makers with HCC in a group of south Brazilian patients. These results may contribute to characterization of HCC profile in Brazil and to provide new data of tissue expression of those markers.

METHODS

Sample characterization

In this case-control study, hepatic specimens contained in paraffin blocks were collected (KCM laboratory) from patients with diagnosis of HCC (case group) and without HCC diagnosis (control group) from Complexo Hospitalar Irmandade Santa Casa de Misericórdia de Porto Alegre (ISCMPA), Brazil. Control group included patients without diagnosis of HCC, but with others hepatic diseases such as non-alcoholic steatohepatitis (NASH), hemosiderosis, hemochromatosis, colelitiasis, autoimmune hepatitis, primary sclerosing cholangitis and primary hyperoxaluria. Informed consent was previously applied to patients and its data of age, gender and etiological and clinical factors were collected from medical records. Liver specimens were obtained after procedures as hepatectomy, liver transplantation or liver biopsy. Samples with excess of necrosis and with an insufficient amount of liver tissue were excluded. The present study was approved by UFCSPA's Ethics Committee (protocol number 1785/2012).

Immunohistochemistry assay

Sections with 4µm of hepatic specimens were prepared and submitted to assay. First, the slides were submitted to deparaffinization for 30 minutes in 60ºC stove, followed by two times dewax in xylene, hydration by passage through graded series of ethanol and wash with distilled water. Antigen retrieval was performed using sodium citrate solution pH 6 for 40 minutes in water-bath at 92ºC. Endogenous

peroxidase activity was blocked with 5% hydrogen peroxidase consisting in three rounds of 10 minutes. After this, the sections were incubated in BSA 1% for 1 hour[38].

Slides were, respectively, covered with mouse anti-TNFα monoclonal antibody (1:300 dilution, Santa Cruz Biotechnology, INC.), mouse anti-NF-κB monoclonal antibody (1:200 dilution, Santa Cruz Biotechnology, INC.), rabbit anti-COX-2 monoclonal antibody (1:50 dilution, Biocare Medical®) and mouse anti-Estrogen receptor monoclonal antibody (1:50 dilution, Abcam®) for 60 minutes and then stayed overnight in 4ºC. After washing with PBS, slides were covered with secondary antibody, incubated for 40 minutes and washed again. Tertiary antibody followed same procedure. MACH 4 Universal HRP-Polymer (Biocare Medical®) was used for this steps. Slides were stained with diaminobenzidine chromogenic system (DAKO®) for 5 minutes, washed, counterstained with hematoxylin and mounted. Positive and negative controls followed same steps as patients' samples. Primary antibody was replaced by PBS for negative control[38].

Image acquisition and analysis

Image acquisition was performed using a camera lucida coupled to an optic microscope (Olympus BX-41, Japan). For each patient were captured four photos of representative fields ("Hot Spots") by 2070x1548 resolution and 24bit, using 200x magnification. Representative fields covered areas with most intense positive staining in hepatocytes (modified protocol of Weidner et al. [39]).

Evaluation of ER nuclear staining was performed by counting positive nucleus using Cell Counter Plugin of ImageJ 1.46b software. Nucleus were counted considering that they were inside the previously defined test-area, unless one of the 2 borderlines of the frame was superimposed over them (forbidden lines), following modified protocol described by Mandarim-de-Lacerda[40].

In turn, for markers NF-κB, TNF-α and COX-2, two parameters were evaluated in each photo: percentage and intensity of stained area (cytoplasmic staining). ImageJ 1.46b software was also used for this evaluation using tools as

Color Threshold, Color Deconvolution (from DAB) and Binarization for percentage analysis and Color Threshold, Color Deconvolution (from DAB) and Measure of Mean Gray value for intensity analysis. In this case, Mean Gray Value was discounted from 255 pixel value. The protocols were modified based on techniques described by Chodbury et al. [41], Brey et al. [42] and Ruifrok & Johnston[43]. A mean of the four photos was calculated for each patient.

Statistics

Statistical analyses were performed using IBM® SPPS Statistics software, version 20. Kolmogorov-Smirnov normality test were used in numeric variables. To test difference between case and control groups, the following tests were used: Chi-Square test for ER expression categories; Student's t test for COX-2 and NF-κB expression; Mann-Whitney U test for TNF-α and NF-κB (intensity parameter) expression. Significance level was considered as $p<0.05$.

RESULTS

Patients' characterization

Table 1 represents characterization of sample, divided in case and control groups. Data from 143 patients were analyzed and was observed a similar average age between case and control groups, that is around fifth decade of life. It was also found a predominance of male sex in both groups as well as cirrhosis and infection with HCV as etiologies most found, followed by infection of HBV and alcohol consumption.

ER expression

In figure 1 is demonstrated a digitized photomicrograph of positive nucleus for ER immunohistochemical staining in case and control liver samples. Table 2 shows percentage of positive nucleus of sample for case and control groups, however no significant difference was found in both groups when compared ($\chi 2=4.962$, $p=0.175$).

COX-2, NF-κB and TNF-α expression

Figure 2 shows (A and B) positive staining of COX-2 marker in cytoplasm of cells. When percentage of stained area parameter (C) was analyzed, we found a significant increase of this protein expression when case and control groups were compared ($t(48)=2.040$, $p=0.04$). However, no significant difference was found for intensity of stained area parameter (D) in both groups when compared ($t(48)= 0.258$, $p=0.798$).

Staining for NF-κB marker was detected in hepatocytes cytoplasm of patients (Fig. 3). No significant difference was found in both percentage and intensity parameters evaluated for both groups when compared ($t(50)= 1.034$, $p=0.430$; $U=326.00$, $p=0.890$; respectively).

TNF-α staining was also cytoplasmic (Fig.4) and a significant difference was found in intensity of stained area parameter when comparing cases and controls ($U= 243.00$, $p=0.03$). No significant difference was found for percentage of stained area parameter in both groups when compared ($U=312.00$, $p=0.368$).

DISCUSSION

HCC is the main type of primary liver cancer worldwide with a higher incidence in men[1,30]. Major risk factors for HCC include conditions such as cirrhosis, hepatitis virus infection and alcohol consumption [1,3,5]. Previous studies showed that cirrhosis is present in 80% of HCC cases[5] while HBV infection is associated with 50%, mostly in East of Asia[1,6,44]. In the present study we evaluated characteristics such as sex, age and etiology in patients with HCC and other liver diseases of a group of patients from south Brazil.

Our results show that the majority of HCC cases are male and in fifth decade of age, which is consist with previous findings[30] that indicate an incidence in men higher than in women, with a risk of HCC 2 to 7 times higher[30,45]. Possible explanations for this is the possibility of men being more predispose to risk factors, the effect of estrogen in inflammation and of testosterone in promoting hepatocytes

proliferation[4,30,45]. This disparity in incidence between men and women emphasizes the importance of studies that associates the effect of sex hormones in development of HCC.

The main etiology found in our sample (around 80% of cases), differently from general incidence, was not HBV infection, but, indeed, HCV infection. In fact, we found no significant difference between groups for HBV infection. Worldwide, HBV infection is associated with at least 50% of HCC cases[1,5,6] which differs from our results that show a higher frequency of HCV infection related to HCC. Our findings, however, are consistent with epidemiologic data of viral hepatitis in south Brazil that shows higher frequency of HCV cases when compared to HBV[46]. Nevertheless, one possible explanation of a lower participation of HBV infection as an etiology in HCC cases in south Brazil is because of the wide range of vaccination against the virus in the region[47,48]. Our findings may help in characterization of, at least, part of brazilian population with a major risk factor different from other regions of the world. Therefore, pointing out the necessity of enhancing different strategies of health promotion and prevention against HCV and the significance of new studies correlating HCV role in development of conditions that lead to liver cancer.

Cirrhosis was also an important finding, over again, consistent with literature, in the majority of HCC patients. However, it was not found a significant difference of frequency between case and controls groups, which means that we can't associate for sure the process as pre-malignant condition, whose mechanism may occur by promoting liver susceptibility [49]. These findings show that cirrhosis may be involved with liver carcinogenesis but also with pathogenesis of other liver diseases, since it was present in 84% of control patients.

Alcohol consumption is another risk factor for HCC, having a minor contribution compared to viral hepatitis [2,4]. Still, in our study, we found a significant difference between groups with a higher frequency of alcohol in control patients. This finding may indicate that alcohol consumption is associated with other liver diseases than HCC. In fact, literature points out that the contribution of alcohol in

HCC is involved in progression of fibrosis, a process that is also associated with other hepatic diseases [2,4,5].

Estrogen seems to have an important role in carcinogenesis of liver, being already demonstrated, in animal models, its part in promoting and inducing hepatocarcinogenesis[32]. On the other hand, in review of Shizumu et al. [50], it is reported that estrogen, a potent antioxidant, may have a protective effect since HCC frequency is higher in men and postmenopausal women, which are individuals who have a decrease in estrogen levels. In previous study of Vizoso et al. [51], it was investigated ER expression in HCC samples by tissue microarray and immunohistochemistry techniques, and it was reported an elevated expression of the protein. Hong et al. [52], in turn, reported an increased susceptibility to DEN-induced HCC initiation and progression in estrogen receptor alpha knockout mice. However, in our study, we didn't find a significant difference in ER immunohistochemistry expression between case and control groups, highlighting the need to further study with a greater number of patients in sample and subset analysis to clarify the contribution of ER expression in HCC. It is import to note that, as a limitation of our study, the hepatic specimens slides were made from paraffin blocks already stocked in prior laboratory of origin, which could have caused difficulty in tissue staining.

Another important point to be discussed is inflammation that seems to have a significant role in promoting conditions that lead to cancer[7]. COX-2, as an inflammation mediator, is related to formation of carcinogens, tumor promotion and other roles in carcinogenesis[22,23]. In the present study, we evaluated the COX-2 expression in patients with and without HCC. HCC patients presented a significant difference of COX-2 cytoplasmic expression when compared to controls. This finding is consistent to other studies in literature that evaluated HCC samples as well as others liver diseases like nonalcoholic-steatohepatitis (NASH), chronic hepatitis and liver cirrhosis[35,36,26].

Our findings emphasize that the study of COX-2 inhibitors could lead to better understanding of COX-2 role in hepatocarcinogenesis and the effects that its inhibition may cause in the development of disease. In fact, some studies already

demonstrated that Celecoxib® (a COX-2 inhibitor) alone caused growth inhibition of human hepatocellular carcinoma cells *in vitro* and *in vivo*[53], as well as by synergistic use with Sorafenib® [54].

Another important inflammatory marker is NF-κB that appears to have dual role in development of HCC due its anti-apoptotic effect: it can either prevent early hepatocytes death but also enhance tumor cells survival as reported in review of Szabo & Lippai[31]. In our study, we analyze the expression of NF-κB in patients with HCC and no significant difference was found between the case and control groups. This result differs from previous study of Jin et al. [37] that analyzed malignant and benign tissue of 305 patients with HCC. Divergence of result may be caused by the size of sample, evidencing the need of further studies of this marker. Still, is also important to highlight that most of our samples showed cytoplasmic staining rather than nuclear one. NF-κB is a nuclear protein factor that stays in inactive form in cytoplasm, being transported to nucleus when it's active[11,13]. Our finding suggests an inactivity of the protein in analyzed sample that could indicate the use of different intracellular pathways for inflammation process that do not use NF-κB as a transcription factor in liver.

TNF-α is a pro-inflammatory cytokine known by its anti-carcinogenic effects, but also associated with pro-tumorigenic properties[15,16]. Our finding showed a significant difference in intensity staining of cells, but not in its percentage of stained area, in control group when comparing to case group. Wang et al. [55] found that TNF-α is highly expressed in HCC *in vitro* cells lines and HCC tumor tissues. The cited study also performed a survival analysis that showed a shorter survival time of these patients compared to low TNF-α expression ones. On the other hand, in study of Dong et al. [56] performed with Sprague–Dawley male rats, TNF-α presented a dual role in CCl4-induced liver injury. In that case, both high and low doses of TNF-α showed an increase of serum levels of alanine aminotransferase (ALT) and aspartate transaminase (AST) enzymes. This dose-related effect of TNF-α could be a possible process by which HCC patients in our study presented lower expression of TNF-α cytokine.

Our findings bring more information of south Brazilian population with HCC, helping to characterize the profile of disease in region. We also bring new information of immunohistochemistry expression of inflammatory and estrogenic makers in human liver tissue that may contribute to new studies in the area. An inherent limitation to our study is the small sample size, which may not have enough power to detect an association of markers as well as difficulty of performing further analysis. Furthermore, the difficult of obtaining samples from healthy patients to use as control it was another limitation of our study that should be pointed.

Table 1. Characteristics of the sample

Variables	Case	Control	*p*
Patients – n	68	75	
Gender – n(%)			0.417
Female	21 (30.9)	28 (37.3)	
Male	47 (69.1)	47 (62.7)	
Age (years) – Mean ± SD	55.14 ± 9.32	55 ± 9.54	0.211
Etiology – n(%)			
Cirrhosis	49 (72)	63 (84)	0.08
HCV	58 (85.3)	39 (52)	>0.001
HBV	3 (4.4)	10 (13.3)	0.06
Alcohol	12 (17.6)	26 (34.7)	0.02

Abbreviation: HCV, Hepatitis C Virus. n: number of patients

Continuous data are shown as mean ± standard deviation and categorical values are shown as absolute frequency (relative frequency).

Continuous data were compared by Mann-Whitney U test and categorical data by the chi-square test.

Table 2. Percentage of positive nucleus for ER stanning in case and control.

	ER		
	Case	**Control**	***p***
0-25%	20 (54%)	18 (60%)	0.175
25-50%	5 (13.51%)	8 (26.6%)	
50-75%	9 (24.32%)	4 (13.4%)	
>75%	3 (8.7%)	0 (0%)	

Categorical values are shown as absolute frequency (relative frequency) and were compared by chi-square test.

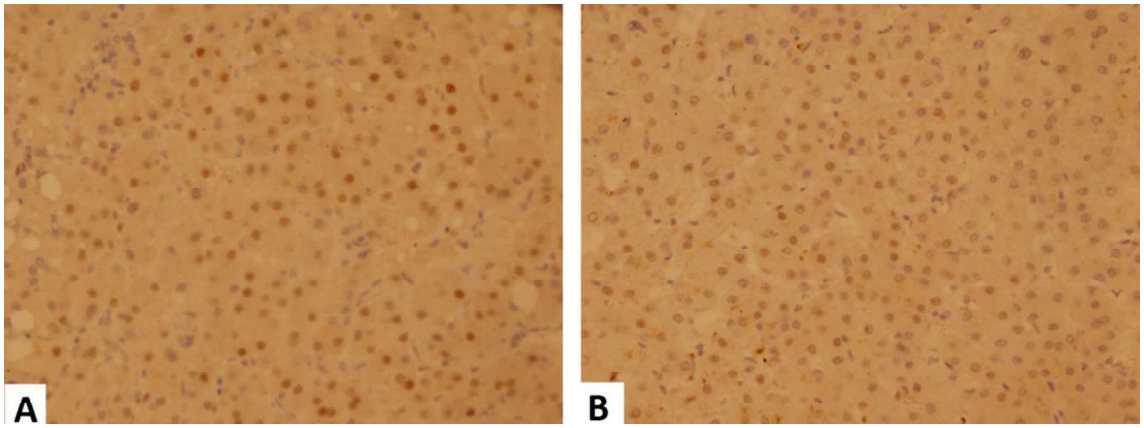

Figure 1. Digitized photomicrograph of positive nucleus for ER immunohistochemical staining in case (A) and control (B) liver samples (original magnification, x200).

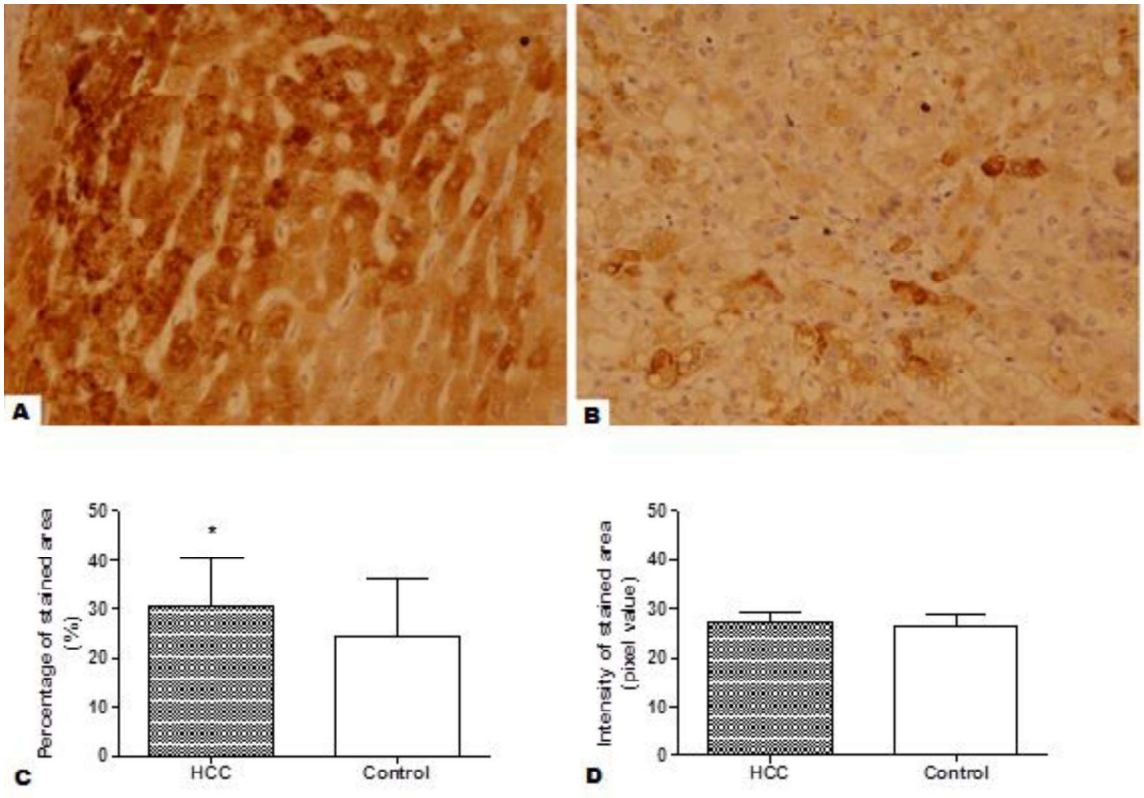

Figure 2. Digitized photomicrograph of immunohistochemical cytoplasmic staining of COX-2 marker in case (A) and control (B) liver samples (original magnification, x200). Mean (±SD) percentage (C) and intensity (D) of stained area for COX-2 maker in case (HCC; n=25) and control (non-HCC; n=25). The Student t test was used to compare case and control groups, at significance level of $p<0.05$. *Indicates a significant difference between groups.

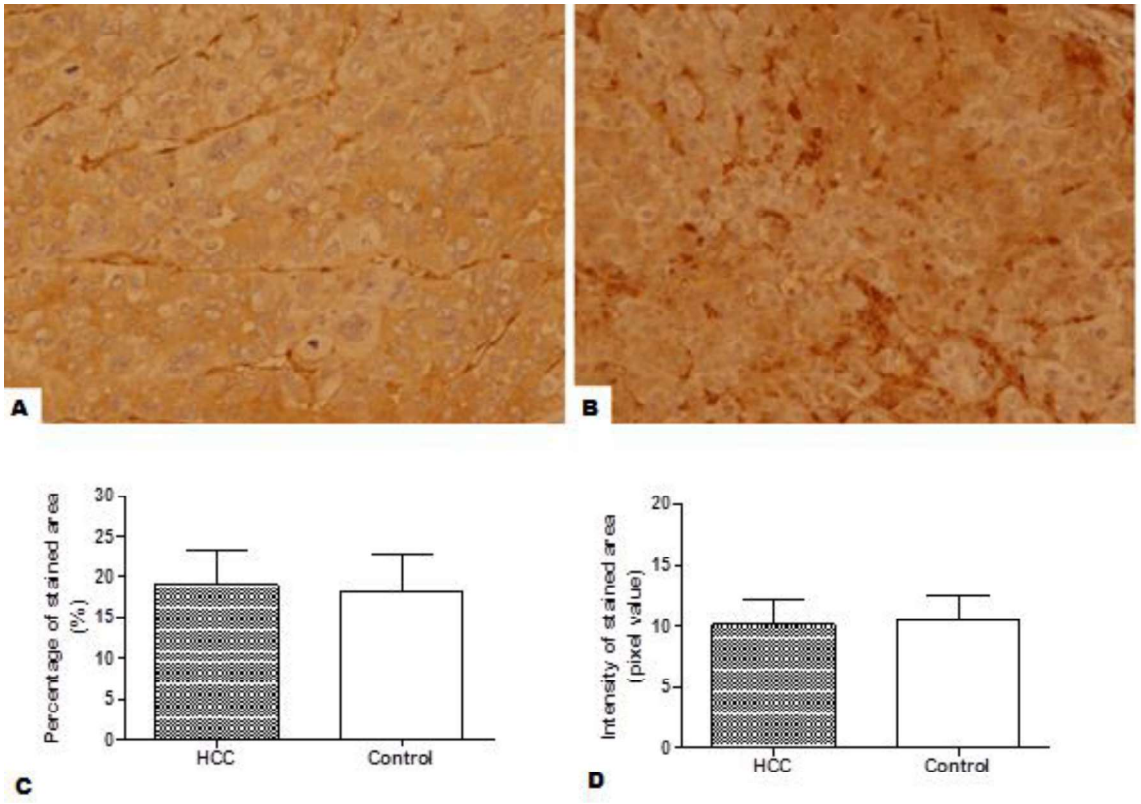

Figure 3. Digitized photomicrograph of immunohistochemical cytoplasmic staining of NF-kB maker in case (A) and control (B) liver samples (original magnification, x200). Mean (±SD) percentage (C) and intensity (D) of stained area for NF-kB marker in case (HCC; n=23) and control (non-HCC; n=29). The Student t test was used to compare case and control groups for percentage parameter and the Mann-Whitney test was used for intensity parameter, at significance level of $p<0.05$.

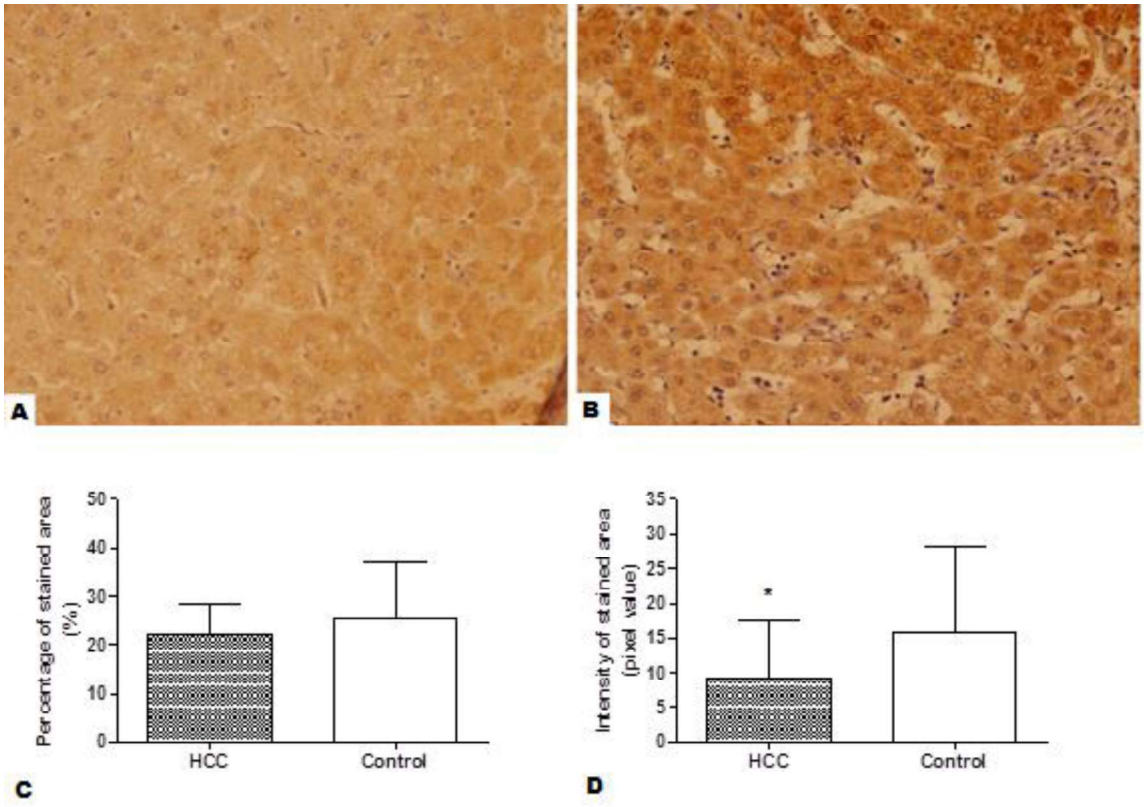

Figure 4. Digitized photomicrograph of immunohistochemical cytoplasmic staining of TNF-α marker in case (A) and control (B) liver samples (original magnification, x200). Mean (±SD) percentage (C) and intensity (D) of stained area for TNF-α marker in case (HCC; n=26) and control (non-HCC; n=28). The Mann-Whitney test was used to compare case and control groups, at significance level of $p<0.05$. *Indicates a significant difference between groups.

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5. CONCLUSÃO

A COX-2 e TNF-α foram os marcadores que apresentaram diferença significativa entre os grupos estudados, configurando-os como marcadores elegíveis de serem estudados de forma mais aprofundada em relação ao perfil imuno-histoquímico e ao papel deles no desenvolvimento do CHC. Além disso, o perfil de fatores de risco dos pacientes com CHC no sul do Brasil parece diferir de outras regiões do mundo na questão do tipo de vírus da hepatite mais associado com a doença. A maior frequência de casos com VHC em pacientes do CHC aponta para uma maior necessidade de programas de prevenção contra a transmissão desse vírus na região sul do Brasil, assim como a realização de estudos mais direcionados em associar o papel do vírus no desenvolvimento do CHC e outras doenças hepáticas.

ANEXO A - Parecer do Comitê de Ética da UFCSPA

Parecer Consubstanciado de Projeto de Pesquisa

Título do Projeto: Determinação de polimorfismos e isoformas nos genes ESRA e ESR2 em pacientes com carcinoma hepatocelular

Pesquisador Responsável Márcia Giovenardi PARECER 1785/12

Data da Versão	**Cadastro 031/12**	**Data do Parecer 11/07/2012**

Grupo e Área Temática III - Projeto fora das áreas temáticas especiais

Sumário do Projeto
Trata-se de um estudo transversal descritivo, no qual serão utilizadas 160 espécimes hepáticos obtidos de pacientes com diagnóstico de CHC e 160 espécimes hepáticos obtidos de pacientes controles, a fim de investigar a variabilidade nos genes dos receptores de estrógenos nestes pacientes

Itens Metodológicos e Éticos	**Situação**
Título	**Adequado**
Autores	**Adequados**
Local de Origem na Instituição	**Adequado**
Projeto elaborado por patrocinador	**Não informado**
Aprovação no país de origem	**Não necessita**
Local de Realização	**Própria instituição**
Outras instituições envolvidas	**Não**
Condições para realização	**Adequadas**

Comentários sobre os itens de Identificação

Introdução	**Adequada**

Comentários sobre a Introdução

Objetivos	**Adequados**

Comentários sobre os Objetivos

Pacientes e Métodos	
Delineamento	**Adequado**
Tamanho de amostra	**Total 160 Local**
Cálculo do tamanho da amostra	**Adequado**
Participantes pertencentes a grupos especiais	**Não**
Seleção eqüitativa dos indivíduos participantes	**Adequada**
Critérios de inclusão e exclusão	**Adequados**
Relação risco- benefício	**Adequada**
Uso de placebo	**Não utiliza**

Período de suspensão de uso de drogas (wash out)	**Nâo utiliza**
Monitoramento da segurança e dados	**Adequado**
Avaliação dos dados	**Adequada – quantitativa**
Privacidade e confidencialidade	**Adequada**
Termo de Consentimento	**Adequado**
Adequação às Normas e Diretrizes	**Sim**

Comentários sobre os itens de Pacientes e Métodos

Cronograma	**Adequado**
Data de início prevista	**ago/2012**
Data de término prevista	**dez/2016**
Orçamento	**Adequado**
Fonte de financiamento externa	**Agência de fomento**

Comentários sobre o Cronograma e o Orçamento

Referências Bibliográficas	**Adequadas**

Comentários sobre as Referências Bibliográficas

Recomendação

Aprovar

Comentários Gerais sobre o Projeto

Cronograma: até 2016 (renovação durante o período?)

ANEXO B - Normas da Revista

O artigo presente nessa dissertação segue as normas da revista World Journal of Gastroenterology (WJG):

1 WRITING REQUIREMENTS

All contributions should be written in English. All articles must be prepared by Word- processing Software, using 12 pt Book Antiqua font and 1.5 line spacing with ample margins. Required information for each of the manuscript sections is as follows:

1.1 Title. The title should be no more than 12 words. The title should summarize the core content of the manuscript, so that the reader may readily understand the key concepts and important findings presented within. This type of succinct and impactful statement will serve to catch readers' attention and stimulate their interest in reading the abstract and/or downloading the full paper. It is also strongly recommended that the title include one or two of the key words associated with the manuscript's topical content, to facilitate the paper being readily found by electronic searches of public databases, such as Google or PubMed. Finally, a succinct and impactful title will include minimal nonfunctional words, such as "a," "an," "the," "roles of," etc. and will avoid non-standard abbreviations.

1.2 Running title. A short running title of no more than 6 words should be provided. It should state the topic of the paper.

1.3 Authorship. Authorship credit should be given in accordance with the standard proposed by the ICMJE (http://www.icmje.org/), specifically, authorship is merited by

(1) substantial contributions to conception and design of the study, acquisition of data, or analysis and interpretation of data; (2) drafting the article or making critical revisions related to important intellectual content of the manuscript; and (3) final approval of the version of the article to be published. Authors should meet conditions 1, 2 and 3. Please note that the co-first author designation is not allowed for any Baishideng Publishing Group (BPG) published article.

1.4 Institution. Author names (unabbreviated) should be given first (first name and family (sur)name), followed by the complete name of the affiliated institution, city,

province/state, postcode and country. For example, Xu-Chen Zhang, Li-Xin Mei, Department of Pathology, Chengde Medical College, Chengde 067000, Hebei Province, China. In the case that one author represents two institutions, the institutions will be listed separately; for example, George Sgourakis, Department of General, Visceral, and Transplantation Surgery, Essen 45122, Germany; George Sgourakis, 2nd Surgical Department, Korgialenio-Benakio Red Cross Hospital, Athens 15451, Greece.

1.5 Author contributions. The format of this section will be as follows: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; Wang CL, Liang L and Fu JF wrote the paper.

1.6 Supportive foundations. Authors are strongly encouraged to submit their high quality manuscripts describing research performed with support by various foundations (i.e., charitable, not-for-profit organizations) to the open-access journals published by the BPG. If the manuscript is accepted for publication, according to the results of peer-review, the authors should provide a copy of the full approved grant application form(s), consisting of the information section and body section, to the BPG in PDF format. The approved grant application form(s) will be released online together with the manuscript in order for readers to obtain more information about the study and to increase the likelihood of subsequent citation. Our purpose of publishing the approved grant application form(s) is to promote efficient academic communication, accelerate scientific progress in the related field, and improve productive sharing of research ideas.

Supportive foundation acknowledgment: The complete name(s) of supportive foundation(s) and identification number(s) of grants or other financial support will be provided on the title page of all submitted manuscripts using the following format: Supported by the National Natural Science Foundation of China, No. 30224801.

1.7 Institutional review board statement. Any article describing a study (basic research, clinical research, and case report) involving human and/or animal subjects

is required to have the institutional review board (IRB) name, whether institutional (part of the author(s)' academic/medical institution, such as the Oak Grove Children's Hospital Institutional Review Board) or commercial/independent/private (contracted for-profit organizations, such as the ClinicCare Coalition for Human Rights Institutional Review Board), stated explicitly on the title page. In addition, a copy of any ethical approval document(s)/letter(s) or waiver should be provided to the BPG in PDF format.

Sample wording: The study was reviewed and approved by the [Name of Institution or Organization] Institutional Review Board.

1.8 Informed consent statement. Any research article describing a study (clinical research and case report) involving humans should contain a statement in the title page clearly stating that all involved persons (subjects or legally authorized representative) gave their informed consent (written or verbal, as appropriate) prior to study inclusion. In general, the BPG requires that any and all details that might disclose the identity of the subjects under study should be omitted or anonymized. In the rare situation that a study participant's identifiable information is crucial to the case presentation, the statement of informed consent is absolutely necessary, unless the participant is deceased. In addition, a copy of any approval document(s)/letter(s) or waiver should be provided to the BPG in PDF format.

Sample wording: All study participants, or their legal guardian, provided informed written consent prior to study enrollment

Waiver of informed consent for human study subjects may be justifiable under certain rare and specific conditions, such as for a trial with demonstrated minimal risk or cases of emergency care. Authors may petition BPG for waiver of informed consent, but there is no guarantee that the petition will be granted. In general, BPG favors the requirement of informed consent for all reports of information (anonymized or identifiable) and reserves the right to refuse publication of such if informed consent was not obtained.

1.9 Conflict-of-interest statement. A conflict-of-interest statement is required for all article and study types. In the interests of transparency and helping reviewers to assess any potential bias in a study's design, interpretation of its results or

presentation of its scientific/medical content, the BPG requires all authors of each paper to declare any conflicting interests (including but not limited to commercial, personal, political, intellectual or religious interests) in the title page that are related to the work submitted for consideration of publication. In addition, reviewers are required to indicate any potential conflicting interests they might have related to any particular paper they are asked to review, and a copy of signed statement should be provided to the BPG in PDF format.

Sample wording: [Name of individual] has received fees for serving as a speaker, a [position; such as consultant and/or an advisory board member] for [name(s) of organization(s)]. [Name of individual] has received research funding from [name(s) of organization(s)]. [Name of individual] is an employee of [name(s) of organization(s)]. [Name of individual] owns stocks and/or shares in [name(s) of organization(s)]. [Name of individual] owns patent [patent identifier information (including patent number, two- letter country code, and kind code) and a brief description].

1.10 Data sharing statement. Basic research and clinical research studies require a data sharing statement. The data sharing statement will be provided in the title page, and will be presented in the form as shown in the sample below. In addition, a copy of the signed statement should be provided to the BPG in PDF format.

Sample wording: Technical appendix, statistical code, and dataset available from the corresponding author at [email address or URL]. Participants gave informed consent for data sharing [or ...consent was not obtained but the presented data are anonymized and risk of identification is low... or consent was not obtained but the potential benefits of sharing these data outweigh the potential harms because...]". If no other data, please state: No additional data are available.

1.11 Correspondence to. Only one corresponding author is allowed. The corresponding author's contact information will be provided in the following format: Author names (unabbreviated) should be given first (first name and family (sur)name), followed by the author's title, affiliation, the complete name of institution, city, province/state, postcode, country and email. All the letters in the email address should be typed in lowercase, and separated from the country by a period and a space. For example, Andrzej S Tarnawski, MD, PhD, DSc (Med), Professor of

Medicine, Chief, Gastroenterology, VA Long Beach Health Care System, University of California, Irvine, 5901 E Seventh St, Long Beach, CA 90822, United States. astarnaw@uci.edu

1.12 Telephone and fax. Telephone and fax numbers should consist of +, country number,district number and telephone or fax number; for example, Telephone: +86-10-85381892 Fax: +86-10-85381893

1.13 Abstract. An informative, structured abstract of no less than 246 words should accompany each manuscript. The Abstract will be structured into the following sections and adhering to the word count thresholds indicated in parentheses:

AIM (no more than 20 words): The purpose of the study should be stated clearly and with no or minimal background information, following the format of: “To investigate/study/determine…”

METHODS (no less than 80 words): You should present the materials and methods used for all of the data presented in the proceeding Results section of the abstract.

RESULTS (no less than 120 words): You should present P values where appropriate. You must provide relevant data to illustrate how the statistical values were obtained, e.g. 6.92 ± 3.86 vs 3.61 ± 1.67, $P < 0.001$.

CONCLUSION (no more than 26 words): You should present your findings and implications that are within the scope of the data you have presented in the preceding Results section. The conclusion should be written in the present tense.

1.14 Keyword. Please list 5-10 keywords for each paper, which reflect the content of the study and are selected mainly from the Index Medicus. Each keyword is to be separated by a semicolon.

1.15 Core tip. Please write a summary of no more than 100 words to present the core content of your manuscript, highlighting the most innovative and important findings and/or arguments. The purpose of the Core Tip is to attract readers’ interest for reading the full version of your article and increasing the impact of your article in your field of study.

1.16 Citation. The citation contains, authors' names and title. The name of the first author should be typed in bold-faced letters; the family (sur)name of all authors should be typed with the first letter capitalized, followed by their abbreviated first and middle initials. For example, an article by Jae Moon Yoon, Ki Young Son, Chun Sick Eom, Daniel Durrance, Sang Min Park will be written as Yoon JM, Son KY, Eom CS, Durrance D, Park SM. Pre- existing diabetes mellitus increases the risk of gastric cancer: A meta-analysis.

1.17 Main text. The main text contains Introduction, Materials and methods, Results, Discussion, Acknowledgments, Conclusion, Comments (Background, Research frontiers, Innovations and breakthroughs, Applications, and Terminology), and References.

1.18 Biostatistics. Any manuscript describing a study (basic research and clinical research) that used biostatistics must include a statement in the Materials and Methods section affirming that the statistical review of the study was performed by a biomedical statistician. Statistical review is performed before the submission or after peer-review. The author invites an expert in Biomedical Statistics to evaluate the statistical method(s) used in the study, including but not limited to the t-test (group or paired comparisons), chi- square test, ridit, probit, logit and regression (linear, curvilinear, or stepwise) modeling, correlation, analysis of variance, and analysis of covariance. The review by the biomedical statistician is conducted with respect to the following points: (1) Statistical methods are adequately and appropriately described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. The number of observations and subjects (n) is given. Losses in observations, such as drop-outs from the study, are reported; (4) Values, such as ED50, LD50 and IC50, have the 95% confidence limits calculated and have been compared by weighted probit modeling (using the functions described by Bliss and Finney); and (5) The word "significantly" is replaced by its synonyms (if it indicates extent) or the P value (if it indicates statistical significance). Statistical data should be expressed as mean ± SD or mean ± SE. Common statistical expressions contains t-test is expressed as t; F-test is expressed as F; chi-square test is expressed as □2; relative coefficient is expressed as r; degree of freedom is expressed as ; number of samples is expressed as n; and

probability is expressed as P. In addition, a copy of any approval document(s)/letter(s) or waiver should be provided to the BPG in PDF format. In addition, a copy of any approval document(s)/letter(s) or waiver should be provided to the BPG in PDF format.

Sample wording: The statistical methods of this study were reviewed by [name(s) of individual(s)] from [name(s) of organization(s)]…

If a biostatistics editor is employed by the authors, the person's name (first name and family (sur)name), qualifications, and contact information must be submitted to the editorial office in the form of a letter of confirmation of service. If the biostatistics editing was performed by a commercial service provider, the company's name and contact information, including URL and email or phone number, must be submitted to the editorial office in the form of a letter of confirmation of service. The letters of confirmation of service must include the corresponding author's name (first name and family (sur)name) and contact information (email and phone number), and the manuscript title.

1.19 Units. Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 g/L; CO2 volume fraction, 50 mL/L CO2, not 5% CO2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23,243,641 (i.e. 23 million, 243 thousand, and 641) should be written as 23243641, with no commas and no spaces. The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/bpg/g_info_20100725073806.htm.

1.20 Illustrations. Figures must be presented in the order that they appear in the main text of the manuscript (numbered as 1, 2, 3, etc.). All figures must have a detailed figure legend that provides a clear and comprehensive description of the information presented in the figure, so that the reader can understand without having to refer back to any other portion of the manuscript. The figure's title and legend must be presented on a separate page from the figure itself.

It is necessary to keep all elements compiled in a line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. Figure file names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Uniform presentation should be used for figures showing the same or similar contents; for example, “Figure 1 Pathological changes of atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...”

Figures with labels, arrows or other markers, photographs, clinical images, photomicrographs, gel electrophoresis, and the like that include labels, arrows or other markers must be submitted in 2 versions: one version with the markers; and the other without. Provide an explanation for all labels, arrows, or other markers in the figure legend. The figure field in the File Description tab of the manuscript submission form allows for uploading of 2 versions of the same figure.

1.21 Tables. Tables must be presented in the order that they appear in the main text of the manuscript (numbered as 1, 2, 3, etc.). A brief, one-line title must be provided for each table. Detailed legends should not be included under tables, instead having the information presented in the main text where applicable; the information should complement, but not duplicate the text. Use one horizontal line under the title, a second under the column headings, and a third below the Table to be above any footnotes. Vertical line and italics should be omitted.

Please note that tables embedded as Excel files within the manuscript are NOT accepted. Tables in Excel should be copied and pasted into the manuscript Word file. All tables will be located at the very end of your article document. Any tables submitted that are longer/larger than 2 pages will be published as online-only supplementary material.

Tables must be primarily cell-based and fully editable. Do not use the following to organize data or structure the table: (1) Returns (“Enter” key); (2) Tabs; (3) Spaces; (4) Colored text; (5) Cell shading; and (6) Cells within cells. The Software should be Word (preferred), Excel. Don’t allow the graphics, boxes, or embedded tables appear in the manuscript. Don’t allow the parts to appear in the manuscript, if there are some

parts, please make Table 1A and Table 1B into Tables 1 and 2, or combine into one table.

1.22 Notes in illustrations and tables. Data that are not statistically significant should not be noted.

Statistical significance in a figure or table should be denoted using superscripted alphabetical lettering, such that aP < 0.05 and bP < 0.01; non-significant values, i.e., P > 0.05, usually do not need to be indicated. If there are other series of P values, the alphabetical subscripted denotation format is continued, such that cP < 0.05 vs control, dP < 0.01 vs control, eP < 0.05 vs group A, and fP < 0.01 vs group B.

Other notes in tables or under illustrations should be expressed as F1, F2, F3 or sometimes as other superscripted symbols (Arabic numerals). In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, etc., in a specified sequence.

1.23 Abbreviations. Standard abbreviations should be defined in the abstract and in the main body of the manuscript upon first mention in the text. In general, terms should not be abbreviated unless they are used three times or more and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA and mAb, do not need to be defined and can be used directly.

1.24 Italics. Quantities: t time or temperature, c concentration, A area, l length, m mass, V volume. Genotypes: gyrA, arg 1, c myc, c fos, etc. Restriction enzymes: EcoRI, HindI, BamHI, Kbo I, Kpn I, etc. Biological nomenclature: H. pylori, E. coli, etc.

1.25 Acknowledgments. Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

1.26 References. This section includes Coding system, PMID and DOI, Style for journal references, Style for book references, and Format for references (Examples). Specific requirements are as follows:

(1) Coding system

The author should number the references in Arabic numerals according to the citation order in the text. The reference numbers will be superscripted in square brackets at the end of the sentence with the citation content or after the cited author's name. For citation content that is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]." If references are cited directly in the text, they should be included with the direct citation content within the text; for example, "From references[19,22-24], we know that...". Before submitting your manuscript, please ensure that the order of citations in text is the same as in the references section, and also ensure the spelling accuracy of the authors' names. Do not list the same citation twice.

(2) PMID and DOI

Please provide the PMID number, which is the serial number that roots the abstract for that publication into the PubMed index, and the CrossRef DOI® (Digital Object Identifier) name, which is a unique string created to identify a piece of scholarly content in the online environment for each reference in the References section. The PMID number can be found at http://www.ncbi.nlm.nih.gov/pubmed and the DOI name can be found at http://www.crossref.org/SimpleTextQuery/. The numbers will be used in the electronic (E)-version of the manuscript published by the WJG.

(3) Style for journal references

For authors' names, the name of the first author should be typed in bold-faced letters; the family (sur)name of all authors should be typed with the first letter capitalized, followed by their abbreviated first and middle initials. For example, an article by Lian-Sheng Ma and Bo-Rong Pan will be written as Ma LS and Pan BR. The title of the cited article will be written in sentence case. The journal title will be written in its abbreviated form (as shown in PubMed) in italics and followed by the article

publication information (not italicized), including the publication date, volume number (in bold-face numbers), and start page through end page (separated by a hyphen). The PMID and DOI will follow this information and be written as [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

(4) Style for book references

For the authors' names, the name of the first author should be typed in bold-faced letters. The family (sur)name of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials.. The book title will follow the authors' names and not be italicized. The publication information will follow, written as punctuated here: publication number, publication place: publication press, year: start page - end page.