tiple steps beginning with atrophic gastritis that is followed by intestinal metaplasia, dysplasia and carcinoma [2,3]. Diffuse type corresponds to poorly differentiated, infiltrating and non-cohesive tumor cells. Although diffuse type is not characterized by the multiple proceeding steps, this shows more metastatic phenotype with poorer prognosis.

Several genetic alterations are more frequently observed in either subtype of gastric cancer. Overexpression of *ErbB2* is selectively found in intestinal-type tumors and may serve as prognostic marker for tumor invasion [4,5]. *ErbB2* expression level was reported to correlate with lymph node or liver metastasis [6,7]. Significant decrease in the expression of E-cadherin (*CDH1*) has also been described preferentially in diffuse-type gastric cancer ranging from 20% to 90% of frequency [8-10]. The decreased expression of *CDH1* is caused by LOH or hypermethylation. Interestingly, hereditary diffuse gastric cancer is caused by germline mutations of *CDH1* gene [11,12]. In addition, mutation in adenomatous polyposis coli (*APC*) which activates Wnt/β-catenin pathway is predominantly found in intestinal-type gastric cancer [13]. Cyclooxygenase-2 (*COX-2*) that is one of the crucial enzymes to synthesize prostaglandin E_2 is highly up-regulated in intestinal-type cancers compared with diffuse-type ones [14]. These genetic alterations could be used as a hallmark of each type of gastric cancer as well as the histological features.

Genome-wide mRNA expression profiles have identified gene signatures to distinguish intestinal- and diffuse-type gastric cancers. Boussioutas *et al.* [15] reported that the gene signature distinctive for intestinal type exhibits the up-regulation of proliferation markers related to DNA replication, spindle assembly and chromosome segregation. Down-regulated genes in the signature are associated with epithelial differentiation. Jinawath *et al.* [16] also developed another gene signature that is differentially expressed between intestinal-type and diffuse-type cancers with Japanese gastric tumor samples. The intestinal-type signature represented enhancement of cell cycle progression, while the genes associate with extracellular-matrix (ECM) are deregulated in the diffuse type signature. These signatures could provide opportunities of developing biomarkers to diagnose/distinguish the two types in both clinical and preclinical researches.

Transgenic mice that develop gastric tumors present suitable models to decipher gastric tumorigenesis, and identify novel therapeutic targets. We have previously developed several transgenic mice in which prostaglandin E_2 production pathway is highly activated specifically in gastric mucosa. *K19-C2mE* mice expressing *COX-2* and microsomal prostaglandin E synthase-1 (*mPGES-1*) develop inflammation-associated hyperplasia [17]. This was mediated through the recruitment of mucosal macrophages. By crossing the *K19-C2mE* mice with *K19-Wnt1* mice, cooperative effect of Wnt1 and PGE_2 on gastric tumorigenesis was investigated. The *K19-Wnt1/C2mE* mice led to the development of dysplastic gastric adenocarcinoma signifying the importance of the Wnt pathway activation to keep the progenitor cells undifferentiated [18]. To examine the additional effect of the suppression of BMP pathway on the prostaglandin E_2 activation, the compound mice of *K19-Nog/C2mE* were established. The *K19-Nog/C2mE* mice cause the development of gastric hamartomas that are morphologically similar to juvenile polyposis (JP) [19]. Although the detailed histological and hypothesis-based molecular analysis implicated the pivotal role of prostaglandin E_2, Wnt and Nog pathway respectively in gastric tumorigenesis, it remains elusive whether the K19-C2mE and its compound transgenic mice show similarity to intestinal type or diffuse type of human gastric cancers when analyzed by non-biased global expression profile.

In order to identify which types of human gastric tumors (intestinal or diffuse type) the C2mE-related mice are more similar to, we compared expression profile of the two types of human gastric cancer with those of *K19-C2mE*, *K19-Wnt1/C2mE*, and *K19-Nog/C2mE* transgenic mice.

Results

Overall gene expression profiles of transgenic animals

We have previously developed several types of transgenic mice in which prostaglandin E_2 pathway is activated. *K19-C2mE* mice expressing *COX-2* and *mPGES-1* induce hyperplasic gastric tumors. *K19-Wnt1/C2mE* mice in which both Wnt and prostaglandin E_2 pathways are activated cause dysplastic gastric tumors. *K19-Nog/C2mE* mice expressing noggin as well as C2mE develop gastric hamartomas. To provide insight into the molecular mechanism of gastric tumorigenesis, gastric tissues from the transgenic mice and wild-type mice were subject to microarray analysis. Using the Affymetrix GeneChip system, mRNA expression levels were measured for 45,037 probe sets, which represent 21,066 Entrez genes and 5,324 other sequences. Increased expression of introduced gene in each transgenic mouse was observed as reported previously [17-19].

Genome-scale overview of the microarray data revealed that expression changes in the three tumor models of *K19-C2mE*, *K19-Wnt1/C2mE* and *K19-Nog/C2mE* were quite similar, whereas overexpression of Wnt1 only or Nog only led to the expression changes in a small portion of genes (Figure 1). This suggests most of expressional changes in the three transgenic mice were caused by the activation of PGE_2 pathway. Hypergeometric test for gene enrichment showed that the genes involved in wound healing and

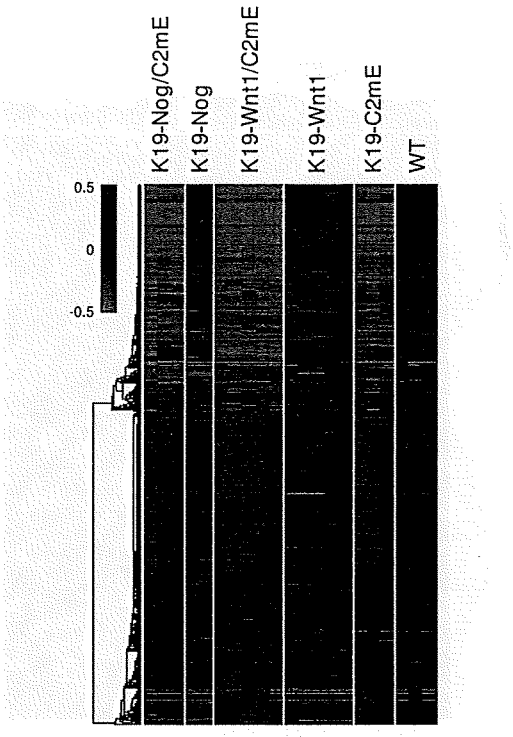

Figure 1
Genome-scale expression pattern of transgenic mice showing major changes are caused by PGE_2 induction. Clustered in rows are 5,440 probe sets selected by fold change threshold of 2 or greater to the average of wild-type and a ratio p-value of 0.01 or less, and columns are mouse gastric samples grouped by genotype. Genotypes are shown on the top of the heatmap. The red-green color scale represents log10 ratio to the average of wild-type samples, as shown in a color bar on top left: red color indicates the gene is up-regulated in the sample, and green indicates down-regulated. WT: wild-type.

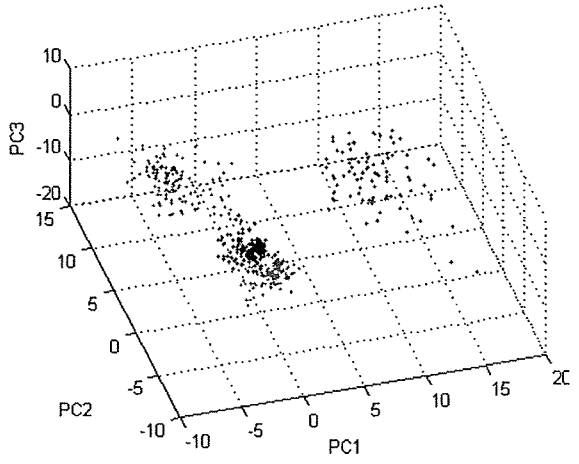

Figure 2
Overall expression changes in gastric tumors of C2mE-related transgenic mice are most similar to those in human gastric cancers. *K19-C2mE*, *K19-Wnt1/C2mE*, and *K19-Nog/C2mE* mouse gastric tumors and human gastric (diffuse, intestinal, and mixed type), colon, breast, and lung cancers were plotted by principal component 1 to 3 (PC1 to PC3) calculated using 1,925 genes which were changed by more than two-fold in more than 50 samples of all. The cumulative contribution of the three components was 32%. Dots shown in blue: human gastric cancers; cyan: human colon cancers; red: human lung cancers; green: human breast cancers; magenta: mouse model tumors.

inflammatory response were significantly condensed with the p-value of 1.5×10^{-21} and 4.2×10^{-13}, respectively, in the gene set changed by the C2mE induction.

Classification of mouse tumor models under a human gastric cancer subtype

In order to confirm that the mouse gastric tumor models are similar to human gastric cancer, the expression profiles were compared with those of human cancer samples. First, gene expression data of human breast, lung, colon, and gastric tumors were collected from public domain. To estimate similarity between the mouse gastric tumors and the four types of human cancers, supervised classification of principal component analysis (PCA) was conducted using 1,925 genes which were changed more than two-fold in more than 50 samples of all human samples. The PCA with the selected genes found that mouse gastric samples from C2mE-related mice were most closely clustered to human gastric cancers among the four tissues examined, indicating the global expression changes in the gastric tumors of the transgenic mice resembled those in human gastric cancers (Figure 2).

Next, in order to examine which subtype of gastric cancer shows cross-species similarity, the mouse tumors were compared with human gastric intestinal-type and diffuse-type cancers on the basis of their expression profiles. Previous expression profiling studies of human gastric tumor samples have identified gene signatures that classify the two types. Intestinal and diffuse types are the two major types of cancer classified on the basis of microscopic morphology [1]. Boussioutas *et al.* [15] showed that proliferation genes were over-expressed in intestinal-type tumors than in diffuse-type tumors; in contrast, extracellular matrix protein genes were up-regulated in diffuse-type compared with intestinal-type tumors. In order to determine which type of human gastric cancer the mouse models are more similar to, we normalized the human data [20] to the average of normal samples, and selected 122 genes which were changed in the opposite direction in

intestinal type and diffuse type [see Additional file 1], to classify intestinal and diffuse types by using the normalized data. The false discovery rate was estimated to be 2.4%. The accuracy of class prediction using this gene set was estimated to be 85% by leave-one-out cross-validation of human samples. We also examined whether this gene set can be used to correctly classify another gastric cancer data set [15]. The test data set included 22 intestinal-type, 35 diffuse-type, and ten normal samples, and was normalized to the average of all normal samples. The error rate was 25% in total, and 29% and 18% in diffuse- and intestinal-type cancers, respectively.

To compare the expression patterns of the signature genes in mouse tumors to those in human gastric cancers, hierarchical clustering analysis was performed with mouse gastric data and human intestinal- and diffuse-type data sets. The expression pattern of our modified signature genes for distinguishing intestinal- and diffuse-type gastric cancers revealed that the gastric tumors from C2mE-related transgenic mice were more similar to intestinal-type human gastric cancers than to diffuse-type human gastric cancers (Figure 3). By linear discriminant analysis, all C2mE-related gastric tumors except one K19-Wnt1/C2mE sample were classified as intestinal-type tumors.

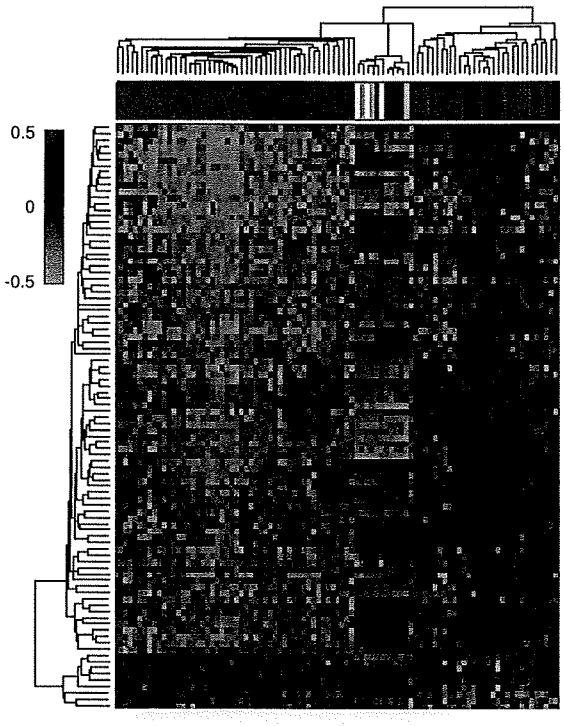

Figure 3
Expression profiles of C2mE-related gastric tumors are clustered to human intestinal-type gastric cancers. Clustered in rows are 93 genes which met p-value less than 0.001 and opposite change direction between intestinal-type and diffuse-type human gastric cancers, and clustered in columns are human and mouse gastric tumors. As a distance measure, cosine correlation was used. Linkage method for clustering was average linkage. Samples shown in red: human intestinal type gastric cancers; blue: human diffuse type; yellow: *K19-C2mE* mice; magenta: *K19-Wnt1/C2mE*: cyan: *K19-Nog/C2mE*. The red-green color scale represents log10 ratio to the average of wild-type or normal samples, as shown in a color bar on top left.

Expression pattern of the genes frequently deregulated in human gastric cancer in a subtype specific manner

It is known that amplification or overexpression of some genes are found in a subtype-specific manner. E-cadherin gene mutations or loss are specifically found in diffuse-type gastric cancer [11,12]. In contrast, amplification of *ErbB2* gene is observed only in intestinal type, and not reported in diffuse type [6,7]. LOH of deleted in colorectal carcinoma (*DCC*) is predominantly observed in about half of intestinal-type [21,22]. Expression levels of the three genes were compared between mice and human gastric cancer types (Table 1). *CDH1* expression was significantly decreased in human diffuse type but not in intestinal type as expected. In the three transgenic mice, *Cdh1* gene was not decreased in any of transgenic mice compared with wild-type, inferring that one of the most characteristic changes in human diffuse type gastric cancer was not observed in the mouse models. Up-regulation of *ErbB2* was observed in human intestinal-type microarray data, and also in our mouse data. *DCC* expression was reduced in human intestinal-type as expected, while the reduction of the gene was observed in the mice model, especially in *K19-Wnt1/C2mE* mice. The expressions of the three genes defining the tissue-type of the human gastric cancer also support the idea that the mouse models are more similar to intestinal-type human cancer.

Difference among PGE_2 pathway-activated mouse models

Tumors from three mouse models with PGE_2 pathway activation show different histology. *K19-C2mE* develops hyperplasia with macrophage infiltration, whereas *K19-Wnt1/C2mE* develops dysplasia [17,18]. *K19-Nog/C2mE* develops hamartoma similar to human juvenile polyposis [19]. We next attempted to identify differentially expressed genes among the three mouse models which allowed us to assess the best-fit model among the three to study gastric intestinal-type cancer. With ANOVA p-value threshold of 0.001, we selected 155 genes which were differently regulated among the three groups. Few of these genes showed expression changes in the same direction between *K19-Wnt1/C2mE* and *K19-Nog/C2mE* (Figure 4). Wnt pathway genes *Porcn*, an acyltransferase required for Wnt protein secretion, β-catenin (*Ctnnb1*), and *Tcfe2a* (*TCF3* in human) were overexpressed in *K19-Wnt1/C2mE*

Table 1: Expression changes of subtype-specific genes in mouse and human gastric tumors.

	Mouse			Human	
	C2mE	Wnt1/C2mE	Nog/C2mE	Diffuse	Intestinal
CDH1	1.13*	1.00	1.10	0.43*	1.09
ErbB2	1.37*	1.43*	1.25*	0.92	1.37*
DCC	0.91	0.85*	0.94	0.98	0.71*

Expression values are shown in average log ratios (base 10) to wild-type or normal samples. Asterisk indicates t-test p-value < 0.05.

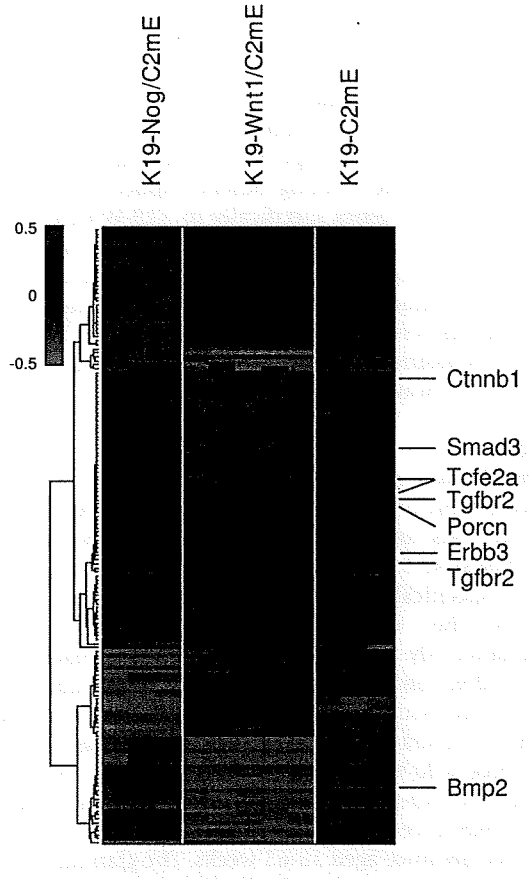

Figure 4
Wnt/β-catenin regulatory genes are up-regulated in Wnt1/C2mE mice. Clustered in rows are 155 probe sets which were differently regulated among three genotypes, *K19-C2mE*, *K19-Wnt1/C2mE*, and *K19-Nog/C2mE*, using ANOVA p-value threshold 0.001. Columns show mouse gastric sample grouped by genotype and genotypes are shown on top of the heatmap. Color scale is same as in Figure 1.

mice, but not in *K19-Nog/C2mE* (Figure 4). TGF-β/BMP pathway genes *Smad3* and *Tgfbr2* were also up-regulated and *Bmp2* was down-regulated in *K19-Wnt1/C2mE* but not in *K19-Nog/C2mE*.

In *K19-Nog/C2mE* mice, some genes which promote tumorigenesis were up- or down-regulated, although they have not been reported in the downstream of BMP pathway. *ROCKII* was specifically up-regulated in *K19-Nog/C2mE*, and its overexpression is associated with progression in several types of cancers via modulating actin cytoskeleton organization. Down-regulated genes include *RAMP2* and *PPARGC1A*, and their inactivation or underexpression was shown to contribute to lung cancer and hepatoma development respectively.

Since deregulation of Wnt pathway including *APC* or *CTNNB1* mutation have been more frequently observed in intestinal-type compared with diffuse-type [23,24], the results indicated that *K19-Wnt1/C2mE* could offer a model that best-fits intestinal-type tumors among the three C2mE-related mice.

Discussion

The present study indicated that human intestinal-type gastric cancers exhibited significant similarity to C2mE-related mice, especially to *K19-Wnt1/C2mE* mice by global expression profiling. The prediction of similar tumor type by global expression profile is consistent with the phenotypes of the transgenic mice. Accumulating evidence has indicated that inflammation level which is caused by the up-regulated expression/activity of *COX-2* and *mPGES-1* is severer in intestinal-type gastric cancer compared with diffuse-type one, although both types of tumors are related to *Helicobacter pylori* that are known to induce inflammation to the infected site [14,25-28]. This knowledge supports our observation that gastric tumors in C2mE-related mice in which PGE_2 pathway is activated exhibit similarity to intestinal-type gastric tumors. In addition, activating and inactivating mutations in *CTNNB1* and *APC* are more frequently observed in intestinal-type cancer. No *APC* LOH/mutation were observed in diffuse-type gastric cancer, whereas 60% were found in intestinal-type one [24,29,30]. Mutation in *CTNNB1* was

predominantly observed in intestinal-type one [13]. This is also concordant with our previous finding that *K19-Wnt1/C2mE* mice which only develop adenocarcinoma among the three C2mE-related mice activate down stream genes of Wnt/β-catenin pathway.

Usually, several types of transgenic mice for one tumor type are required to examine similarity in global expression profiling between mice tumor models and human ones, since the genes which were up- or down-regulated in each mice model were extracted compared to the average of all the examined tumor samples. With this approach, Lee *et al.* [31] analyzed gene expression data of seven mouse hepatocellular carcinomas (HCCs) including five GEMs with human HCCs to identify models that recapitulate human cancer or a type of human cancer, and found that some subclasses of human HCC mimic mice models in expression pattern. Hershkowitz *et al.* [32] also used the same normalization method, and found that characteristic expression patterns observed in human breast tumors were conserved in 13 mouse breast tumor models. Since the available data of expression profile for mouse gastric tumors are limited to our *K19-C2mE* and its compound mice, we took different strategy to assess the similarity of gastric tumors between the two species. Instead of using average of all samples in the dataset as a reference to calculate expression ratios, we normalized the mouse gastric data to average of wild-type samples. To compare our mice expression profiles with those of human gastric cancers, the gene signature to classify human intestinal- and diffuse-type gastric cancers was also modified from original one by normalizing the expression data to the average of normal gastric samples. This has allowed us to reveal that C2mE-related transgenic mice resemble human intestinal-type gastric tumors in expression profiling.

Comparison of gene expressions between mouse models showed that simultaneous induction of Wnt1 and PGE_2 deregulated not only gene expression of *Ctnnb1* and *Porcn* in Wnt signaling but also *Smad3* and *Tgfbr2* in TGF-β/BMP signaling. Given the crosstalk between TGF-β/BMP and Wnt pathways has been reported in multiple previous studies, the deregulated expression of the genes in the additional signaling pathways could be explained by positive and negative feedback to the pathways from the up-regulated Wnt signaling. For example, BMP signaling is known to suppress β-catenin activity in intestinal stem cells [33]. BMP signaling could be repressed in *K19-Wnt1/C2mE*, because *Bmp2* expression was significantly down-regulated. Increase in *Smad3* and *Tgfbr2* might be resulted from the negative feedback by BMP signaling suppression, as demonstrated in a study on TGF-β induced fibrosis [34]. In contrast to *K19-Wnt1/C2mE* transgenic mice, expression changes of the Wnt pathway genes were not observed in *K19-C2mE* and *K19-Nog/C2mE* mice. It would be of great interest to further analyze the crosstalk of signaling pathways in the compound transgenic mice.

Conclusions

Genetically engineered mouse (GEM) models provide useful tools to study mechanism of tumorigenesis, to validate a new target for drug development, and to find biomarkers. Advances in genetic engineering have allowed us to develop a variety of transgenic or knockout models of human diseases. The main question on using GEMs as disease models is whether the model recapitulates the human disease. We previously developed several gastric tumor transgenic mice in which prostaglandin E_2 pathway is activated. Although we conducted detailed histological analysis with the transgenic mice, it remained elusive whether global molecular changes in the transgenic mice reproduce features of human gastric tumors or not. This report has provided initial evidence that *K19-C2mE* and their compound mice, *K19-Nog/C2mE*, *K19-Wnt1/C2mE*, show similarity to human gastric cancer, especially to intestinal-type one by the analysis of mRNA expression profile. Among others, extraction of up- or down-regulated genes specifically in *K19-Wnt1/C2mE* or *K19-Nog/C2mE* respectively inferred that *K19-Wnt1/C2mE* mice would provide best-fit mouse model for intestinal-type gastric tumors. These findings would potentially provide various benefits in our future studies including elucidation of gastric tumorigenesis and optimal therapeutic target identification.

Methods

Stomach tissue samples

Construction of transgenic mice have been described in our previous studies [17-19]. Briefly, the *K19-Wnt1* and *K19-Nog* strains overexpress *Wnt1* and *Nog* genes, respectively, specifically in the stomach. *K19-C2mE* overexpresses the *mPGES-1* gene and *COX-2* genes simultaneously and specifically in the stomach. *K19-Wnt1/C2mE* and *K19-Nog/C2mE* are compound transgenic mice with *K19-Wnt1* and *K19-Nog*, respectively; both mouse strains have *K19-C2mE*. For expression profiling, three wild-type C57BL/6, five *K19-Wnt1*, three *K19-C2mE*, five *K19-Wnt1/C2mE*, two *K19-Nog*, and three *K19-Nog/C2mE* mice were used. All animals used in this study were female mice aged 18-65 weeks. The glandular stomach of each mouse was cut for microarray analysis. All animal studies were carried out in accordance with good animal practice as defined by the Institutional Animal Care and Use Committee (IACUC).

Microarrays

GeneChip Mouse Genome 430 2.0 Arrays (Affymetrix, Inc.) were used to monitor the expression profiles of the gastric samples. Total RNA was prepared using the RNeasy Mini Kit (QIAGEN) after treatment with TRIzol (Invitro-

gen Corp.), and labeled cRNA was prepared using standard Affymetrix protocols. The signal intensities of the probe sets were normalized by the Affymetrix Power Tools RMA method implemented in Resolver software (Rosetta Biosoftware), and log ratio values to the average of wild-type samples were calculated for each sample by using Resolver. All the microarray data were deposited at Gene Expression Omnibus (GEO) under dataset accession no. GSE16902 [35].

Public human microarray data

Human gastric cancer [20] and breast cancer [36] microarray data were retrieved from the online supplement in the Stanford Microarray Database [37]. The gastric cancer data includes 68 intestinal-type cancer, 13 diffuse-type cancer, and 15 normal gastric samples. The breast cancer data include 115 breast tumor and seven normal tissue samples. Human colon cancer data [38], including 100 colorectal cancer and five normal tissue samples, were retrieved from NCBI GEO under accession GSE5206. The Ann Arbor lung tumor dataset [39] including 86 lung adenocarcinomas and 10 non-neoplastic lung samples was obtained from the United States National Cancer Institute website [40]. Expression values were transformed to log10 (ratio to geometric averages of normal samples) in order to compare with mouse data.

Intestinal vs. diffuse type signature genes

Human gastric tumor data from Chen *et al.* [20] were used to develop an intestinal vs. diffuse type classifier. We selected genes that met the following criteria: (1) t-test p-value < 0.001 between the two groups, (2) opposite changes in the average expression of signature genes in intestinal-type tumors and that of signature genes in diffuse-type tumors. The false discovery rate was estimated by the Benjamini and Hochberg method [41]. The tumor classes of mouse and human samples were predicted by linear discriminant analysis using the signature score defined by the following formula:

Signature score = (Average log ratio of genes up-regulated in intestinal-type tumors and down-regulated in diffuse-type tumors) - (Average log ratio of genes down-regulated in intestinal-type tumors and up-regulated in diffuse-type tumors)

Combining mouse and human gene expression data

In order to combine mouse data with human gastric cancer microarray data, mouse and human data were re-ratioed to the geometric average of wild-type and normal samples, respectively. When there was more than one probe set for a gene in a microarray, the averaged expression ratios were used for the gene. Next, using only homologous genes that are represented in both arrays, we merged the mouse and human data sets into a single data set. The mouse microarray contains 45,037 probe sets, which correspond to 21,066 Entrez genes, and the human microarray contains 6,688 probes, which correspond to 4,463 Entrez genes. When they were merged, 4,094 homologous genes were identified.

Statistical analysis

The hypergeometric test for Gene Ontology enrichment was performed using the Gene Set Annotator developed by Rosetta Inpharmatics [42]. For the other statistical analyses in this study, the MATLAB software (MathWorks Inc.) was used.

Authors' contributions

MO and HK designed the research. HO constructed the transgenic animals and prepared the stomach tissue samples. HI analyzed the microarray data and wrote the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

A list of intestinal type vs. diffuse type signature genes. Sequence accession, gene symbol, and the average of log10 ratios in intestinal-type and in diffuse-type, respectively, are shown for each of the 122 cDNAs.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-615-S1.XLS]

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5 Obesity: A risk factor for hepatocellular carcinoma

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Summary

Obesity is increasingly being recognized a risk factor for a number of chronic diseases, including cancer. Insulin resistance is the most widely accepted link between obesity and chronic disease, particularly colorectal carcinoma. In addition to viral hepatitis, obesity/ fatty liver/ non-alcoholic fatty liver disease (NAFLD) may be associated with the morbidity and mortality of hepatocellular cancer (HCC). This review was attempted to understand the relationship between obesity/viral hepatitis and liver cancer. Obesity is associated with the

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risk of death from cancers at individual sites including liver cancer. The condition is an independent risk factor for HCC in patients with alcoholic cirrhosis and cryptogenic cirrhosis. Because NAFLD has been implicated as a major cause of cryptogenic cirrhosis, the development of HCC may be part of progressive nature of this condition. The early diagnosis and effective treatment of fatty liver coupled with HCC are supposed to improve the prognosis of obese patients. Since obesity is associated with the incidence and mortality of HCC, more frequent surveillance for HCC may be warranted in obese patients with fatty liver/NAFLD. Also, attempts should be made to interrupt the progression from simple hepatic steatosis to steatohepatitis, cirrhosis, and advanced HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is a malignant epithelial neoplasm that arises from hepatocytes, the major cell type in the liver. HCC is one of the major causes of death in patients with chronic hepatic disorders, and the disease has recently increased in incidence and mortality in Western countries and Japan [1-5]. The disease is often clinically silent until it is well advanced or tumor diameter exceeds 10 cm. Given the poor prognosis and lack of effective therapies for HCC, prevention programs are desperately needed. The known causes of liver cancer are conditions such as hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, substantial alcohol consumption, and non-alcoholic fatty liver disease (NAFLD). Patients with these conditions often have a spontaneous clinical course associated with chronic hepatitis leading to liver cirrhosis and subsequent liver cancer. In Japan, particularly, there is a higher incidence of liver cancer resulting from HCV infection, and in this context, efforts should be made to reduce the development of this cancer in patients with positive anti-HCV antibodies [6]. To date, various epidemiological and other studies have extensively investigated possible risk factors of liver cancer due to chronic hepatic disorders, identifying sex, age, severity of hepatic inflammation and fibrosis, and race as some of these factors [3, 5, 7, 8], In addition, recent interest in NAFLD has prompted investigation of additional possible risk factors including diabetes mellitus [3, 5, 7, 9-12], obesity as indicated by the body mass index (BMI) [3, 13-15], and hyper-insulinemia [16, 17].

Obesity is known to be one of the most important risk factors for non-alcoholic steatohepatitis (NASH)/NAFLD [18]. In Japan, observed increases in NASH/NAFLD in selected areas or settings may be linked to the increase in overweight and obesity in the last few decades. Obesity could be a major key to explaining NASH/NAFLD, because of the complex causal web that includes obesity, insulin resistance, dyslipidemia, and high blood pressure. Also, obesity is increasingly recognized as a risk factor for a number of gastrointestinal

neoplasms. However, literature on the underlying pahophysiological mechanisms is sparse and ambiguous [19].

The aim of this review is to introduce information regarding epidemiology and risk factors, especially obesity/NAFLD of HCC.

2. Epidemiology

HCC represents approximately 6% of all malignancies. It is the fifth most common malignancy in men and ninth in women, with an estimated 500,000 to 1 million new cases annually around the world [20]. Its incidence is low in the occidental world and high in Southeast Asia and sub-Saharan Africa, even though it has risen in the United States, Japan. England, and France [21]. It is well known that advancing age increases the chances of liver cancer, which occurs most often in people age 50 or older. The HCC rate is 2-4 times higher in men than in women. For example, the cumulative HCC incidence during more than eight years of follow-up in a cohort of 1,927 HCV positive subjects was 21.6% for men compared with 8.7% for women. African-Americans and Latinos appear more likely to develop liver cancer than whites, and the rate among Asians is particularly high due to the high prevalence of chronic hepatitis B in this population. However, the prevalence in young people has risen in recent years due to environmental risk factors at birth [1]. Co-infection of HBV in people with HCV infection elevates HCC development risk. The mechanisms that cause this high incidence include augmented fibrosis, and inflammation and high cellular re-change [22]. The three most important risk factors for HCC development are HCV infection, HBV infection, and cirrhosis caused by alcoholic liver disease. In people with HCV or HBV chronic liver disease, HCC can develop in approximately 10 to 30 years [1]. Some studies have shown that high alcohol consumption (more than 80 g/day) and cirrhosis caused by alcohol consumption are strongly associated with HCC development even in the absence of viral infection [23, 24]. High alcohol consumption and viral hepatitis (primarily HCV infection) represented 63% of HCC cases [23].

3. Genetic alterations

Certain genetic alterations have been associated with HCC development. Table 1 summarizes the most important genes implicated in HCC. p53, localized in chromosome 17p, is mutated in 30% of HCC cases worldwide. This mutation primarily occurs either because of aflatoxins or HCV, HBV chronic infection [25]. A protein is produced by p53 that recognizes injured DNA and controls cell replication [26]. Aflatoxin B_1-8,9-epoxide-aflatoxin is a toxic product of aflatoxin metabolism and is metabolized by the epoxide hydrolase and glutatione *S*-transferase. If this toxin is not metabolized, it combines with genomic structures to create mutations in p53, producing toxic

Table 1. Potential and candidate suppressor genes for HCC.

Potential and candidate suppressor genes	Region
- p73 (functionally related to p53)	1p36
- Potential genes include albumin, alcohol dehydrogenase (ADH3), fibrinogen, and UDP- glucoronyl-transferase	4q
- Insulin-like growth factor 2	6q26-27
- PDGF-receptor beta-like tumor suppressor	8q21-22
- BRCA2 and retinoblastoma gene	13q12-q32
- p53	17p13.1

accumulation [26, 27]. Insulin-like growth factor 2 receptor (IGF2-R) and SMAD4 and SMAD2 genes are also involved in HCC development. The primary function of IGF2-R is the activation of the transforming growth factor beta (TGF-beta) and the SMAD4 and SMAD2 intracellular mediators of the TGF-beta, resulting in growth inhibition and apoptosis [25]. Mutation and chromosomal deletion of IGF2-R occurs in 61% of HCC cases associated with other factors such as viral hepatitis and cirrhosis [26].

4. Risk factors, predisponent conditions and pathogenesis

The major etiologies of HCC are well defined (Table 2). An elevated body mass index (BMI), especially in men [22] and diabetes mellitus [27] are included among the well-known factors. Some of the steps involved in the molecular pathogenesis of HCC have been elucidated in the recent years. As for most types of cancer, hepatocarcinogenesis is a multi-step process involving different genetic alterations that ultimately lead to the malignant transformation of hepatocytes. While significant progress has been made in recognizing the sequence of events involved in other forms of cancer, most notably in colorectal cancer and certain hematopoietic malignancies, the molecular contribution of the multiple factors and their interactions in hepatocarcinogenesis are still poorly understood. HCCs are phenotypically (morphology and microscopy) and genetically heterogenous tumors (Fig. 1a and b), possibly reflecting in part the heterogeneity of etiologic factors implicated in HCC development, the complexity of hepatocyte functions and the late stage at which HCCs usually become clinically symptomatic and detectable. Malignant transformation of hepatocytes may occur regardless of the etiologic agent through a pathway of increased liver cell turnover, induced by chronic liver injury and regeneration in a context of inflammation, immune

response, and oxidative DNA damage. This may result in genetic alterations, such as activation of cellular oncogenes, inactivation of tumor suppressor genes, possibly in cooperation with genomic instability, including DNA mismatch repair defects and impaired chromosomal segregation, overexpression of growth and angiogenic factors, and telomerase activation [28-35]. Chronic HBV-/HCV-hepatitis, alcohol, metabolic liver diseases such as hemochromatosis and alpha1-antitrypsin deficiency as well as NAFLD may act predominantly through this pathway of chronic liver injury, regeneration and cirrhosis. Accordingly, the major clinical risk factor for the development of HCC is liver cirrhosis since 70-90% of HCCs develop into a cirrhotic liver. Most HCCs occur after many years of chronic hepatitis that provides the mitogenic and mutagenic environments to precipitate random genetic alterations resulting in the malignant transformation of hepatocytes and HCC development.

Aflatoxin is a toxin produced by *Aspergillus flavus* and *A. parasiticus*, which grow in foods like peanuts [27]. It is related to HCC in countries where infestation of crops and animal feed is common [30]. Aflatoxin metabolism produces aflatoxin B_1-8,9-epoxide, a toxic product that induces a G to T mutation of the p53 gene at codon 249 up-regulating IGF2 that leads to a reduction of apoptosis and HCC formation [31, 32].

Individual polymorphisms of drug-metabolizing enzymes, such as cytochrome P450 oxidases, *N*-acetyltransferases, and glutathione *S*-transferase, may also contribute to the genetic susceptibility to HCC development [26].

Table 2. Risk factors for HCC development.

Risk factors	
Major	Minor
- Chronic viral hepatitis B/C	- Primary biliary cirrhosis
- Autoimmune hepatitis	- Thorotrast exposure
- Cirrhosis (e.g., HBV+, HCV+, cryptogenic)	- Tobacco
- Hereditary metabolic liver diseases (e.g., hereditary hemochrornatosis, alpha1-antitypsin deficiency, Wilson' s, disease)	- Vinylic exposure
- Glucogenosis	- Estrogen or androgen
- Toxins (e.g., alcohol, Afiatoxin)	
- Overweight (especially in males), diabetes mellitus, nonalcoholic steatohepatitis (NASH) or nonalcoholic fatty liver disease (NAFLD)	

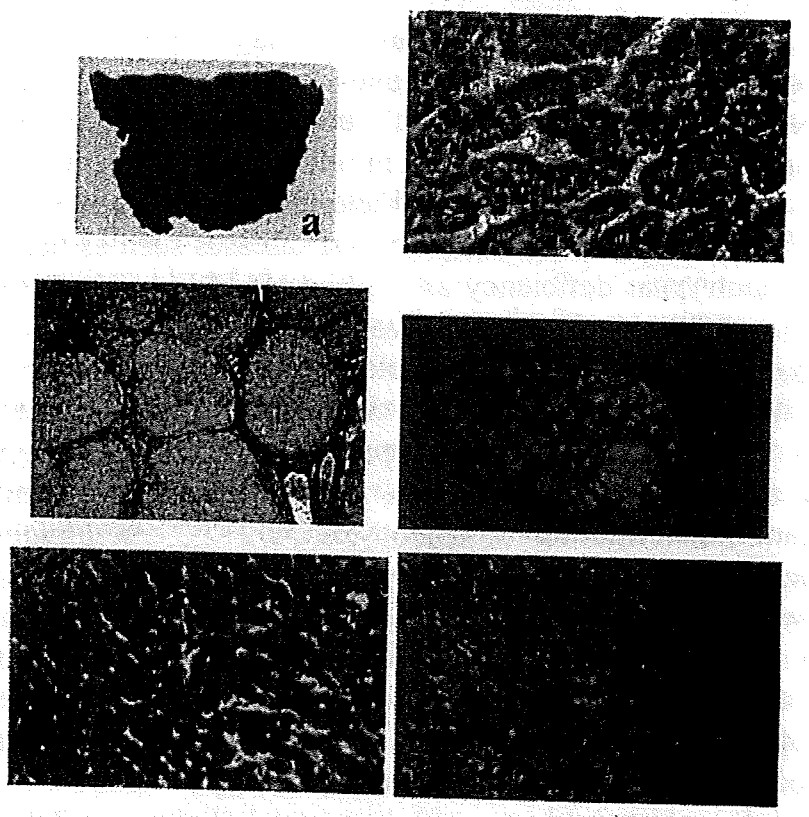

Figure 1. Macroscopic view and histopathology of liver lesions. (a) A small HCC in liver cirrhosis; (b) Trabecular type of HCC (hematoxylin and eosin stain); (c) Liver cirrhosis (Azan-Mallory stain); (d) A large regenerative nodule (circle) in cirrhotic liver; (e) Small-cell dysplasia (hematoxylin and eosin stain); and (f) Large cell-dysplasia (hematoxylin and eosin stain).

5. Liver cirrhosis

Independent of its cause, liver cirrhosis (Fig. 1c) is considered a major clinical and histopathological risk factor for HCC development. Five per cent of all cirrhotic patients develop HCC [27]. We also know that cirrhosis caused by alcohol consumption alone is an important risk factor for HCC development, but alcohol consumption is a co-factor to prior exposure to HBV infection in accelerating HCC development in Japan [24].

The HCC risk in patients with liver cirrhosis depends on the activity, duration and etiology of the underlying liver disease (Fig. 2). Clinical and biological variables (age, anti-HCV positivity, partial thromboplastin time (PTT) and platelet count) allow to further identify a subset of cirrhotic patients with the highest risk of HCC development [36]. Co-existence of etiologies, such as HBV and HCV infection, HBV infection and aflatoxin B_1 [35, 37], HBV/HCV infection and alcohol or diabetes mellitus [38] or HCV infection and liver steatosis [39], increases the relative risk of HCC development. Also,

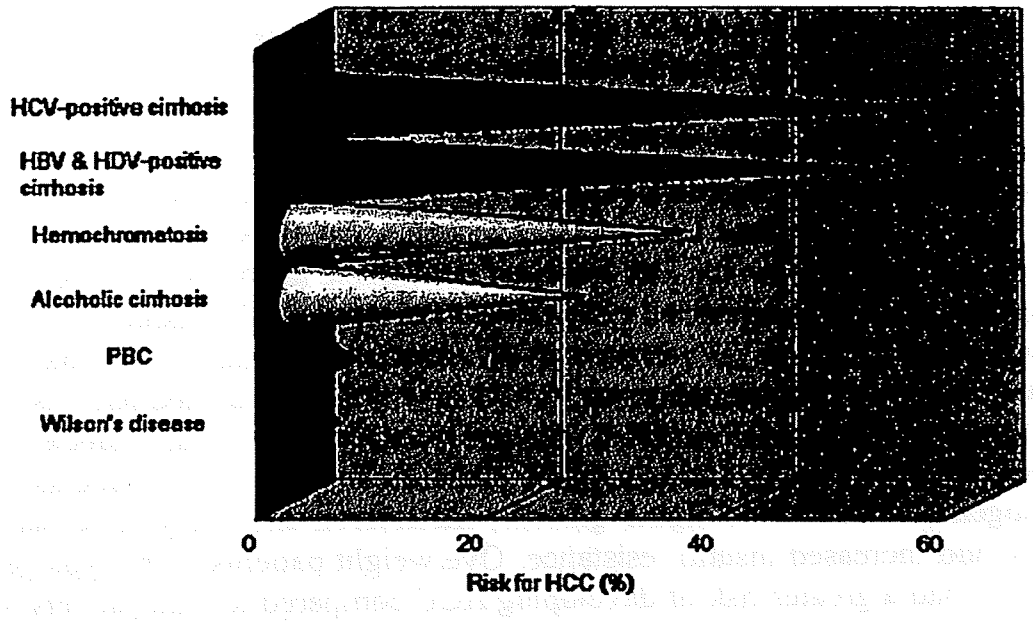

Figure 2. Liver cirrhosis for risk of HCC.

occult HBV infection (anti-HBC positive alone) carries a significant HCC risk [13, 40]. In general, HCCs are more frequent in males than in females and the incidence increases with age. On the other hand, there is evidence that HBV and possibly HCV under certain circumstances play an additional direct role in the molecular pathogenes.is of HCC. Aflatoxins can induce mutations of the p53 tumor suppressor gene, thus pointing to the contribution of an environmental factor to tumor development at the molecular level. Furthermore, in a transgenic mouse model it has been shown that chronic immune-mediated liver cell injury without environmental or infectious agents is sufficient to cause HCC [41] and that inhibition of cytotoxic T lymphocyte-induced apoptosis and chronic inflammation by neutralization of the Fas ligand and prevents HCC development in this model [42]. In addition, in a transgenic mouse model, NF-kappaB may be the link between inflammation and HCC development [25, 43].

6. Cryptogenic cirrhosis

According to a review by Morgan [44], long-term heavy alcohol use (more than 80 g per day for 10 years) increases the risk of HCC by about 5-fold, although studies suggest that light-to-moderate drinking is not linked to a significant increase in liver cancer. Other liver toxins, including tobacco smoke and aflatoxins from moldy grains, may also increase HCC risk.

Ludwig et al. [36] gave the name NASH to an advanced form of fatty liver disease in 1980, defining it as a well-recognized clinical-pathologic syndrome primarily occurring in obese female populations with diabetes mellitus, with histopathological similarities to alcoholic liver disease in the absence of heavy

alcohol consumption. NAFLD affects 10 to 24% of the total population in various countries [37]. This prevalence is higher in high-risk groups with a prevalence of 70 to 86% in obese and/or diabetic patients [38]. NASH is estimated to occur in 10% of NAFLD patients. NASH has been posited as a possible cause of cryptogenic cirrhosis [39]. Patients with cryptogenic cirrhosis also develop HCC. There is increasing evidence that obesity and NAFLD are risk factors for HCC as the link between cryptogenic cirrhosis and NAFLD in many patients is strengthened [13]. Bugianesi et al. [41] conducted a case-control study in which 23 retrospectively identified patients with cryptogenic cirrhosis and HCC were compared to 641 age- and sex-matched patients with alcohol or viral cirrhosis and HCC. The prevalence of obesity and diabetes was higher in the patients with cryptogenic cirrhosis. In addition, the patients with cryptogenic cirrhosis had higher glucose, cholesterol, and triglyceride plasma levels, and increased insulin resistance. Overweight patients with cryptogenic cirrhosis had a greater risk of developing HCC compared to lean patients with cryptogenic cirrhosis [42]. Although NASH may progress to cirrhosis, it is not known if NASH has a role in the development of HCC. Features suggestive of NASH are frequently observed in patients with cryptogenic cirrhosis-associated HCC. Some studies have confirmed that HCC may represent a late complication of cryptogenic cirrhosis in patients with metabolic syndrome [42, 43].

As discussed by Caldwell et al. [45], a growing body of research links HCC to insulin resistance, diabetes, and obesity. For example, people with diabetes had a 3-4 times higher risk of developing liver cancer [46]. This likely occurs because excess fat and blood sugar abnormalities are linked to steatosis (fatty liver), which in turn is associated with worsened fibrosis progression. Given the current epidemic of obesity and diabetes in the United States, this risk factor will be increasingly important [47]. Interestingly, however, Dr. Tanaka's group found that HCC was less likely in individuals with higher blood cholesterol levels [48].

7. Precursor histological injuries

Hepatocarcinogenesis is a multi-step process characterized by the accumulation of poorly understood interacting genetic alterations. HCC co-exists with a number of microscopically distinct lesions that are thought to be its precursors.

Regenerative nodules (Fig. 1d) are characteristic lesions of the cirrhotic liver. They exhibit a lack of bile ducts and poorly organized hepatocytes surrounded by fibrosis and proliferating cholangiocytes. These lesions are arbitrarily classified as micro- or macro-nodular (cut point 0.3 cm). Regenerative nodules may present dysplastic foci, which are smaller than 1 mm and can only be recognized by microscopic studies. There are two types of dysplastic foci in

cirrhotic livers, the small cell-dysplasia (Fig. 1e) and the large cell-dysplasia (Fig. 1f), according to the nucleo-cytoplasmic ratio of each one: high in small cell-dysplasia and normal in large cell-dysplasia. Small cell-dysplasia is thought to be HCC precursor lesions that result from the proliferation of hepatocytes and oval cells. On the other hand, large cell-dysplasia apparently arise from persistent necrosis/inflammation-induced senescent hepatocytes and are therefore not considered to be HCC precursor lesions, although patients with large cell-dysplasia are at an increased risk of HCC [49].

Dysplastic nodules are microscopically recognizable lesions that show atypical features microscopically, such as increased nucleo-cytoplasmic ratio, nuclear contour, thickness of hepatocellular plates and compression of adjacent hepatocytes. Dysplastic nodules represents parts of a spectrum that is arbitrarily divided for the purposes of clinical utility into low-grade or high-grade dysplastic nodules, according to the presence of cytological or structural atypia or both [50]. The risk of HCC in patients with high-grade dysplastic nodules is four-fold higher. By contrast, patients with only low-grade dysplastic nodules are not at a significantly increased risk of HCC [51].

8. Obesity and HCC

Recently, several epidemiological observations have implicated obesity as an independent risk factor for certain malignancies such as endometrial cancer, breast cancer (in post-menopause women), gallbladder cancer (in females), colorectal cancer (predominantly in males), renal cell carcinoma, and esophageal adenocarcinoma. Previously, two studies that examined obesity and liver cancer in Sweden and Denmark found an excess incidence of HCC in obese patients in both men and women, with relative risks in the range of 2.0 to 4.0, and the results of the prospective study in Sweden suggest that the excess risk of death from liver cancer is higher among men than among women [52, 53]. Calle et al. [13] prospectively studied population of more than 900 000 adults in the USA (404 576 men and 495,477 women) who were free of cancer at enrollment in 1982. During 16 years of follow-up, there were 57,145 deaths from cancer. They examined the relation in men and women between the BMI in 1982 and the risk of death from all cancers and from cancers at individual sites, while controlling for other risk factors in multivariate proportional-hazards models. Furthermore, they also suggested that the heaviest members of this cohort (those with BMI>40 kg/m^2) had death rates from all cancers combined, which were 52% higher (for men) and 62% higher (for women) than the rates in men and women of normal weight. BMI was significantly associated with higher rates of death due to cancer of the liver, esophagus, colorectum, gallbladder, pancreas, and kidney (Fig. 3). As compared with men of normal weight, men with BMI>35.0 kg/m^2 had significantly

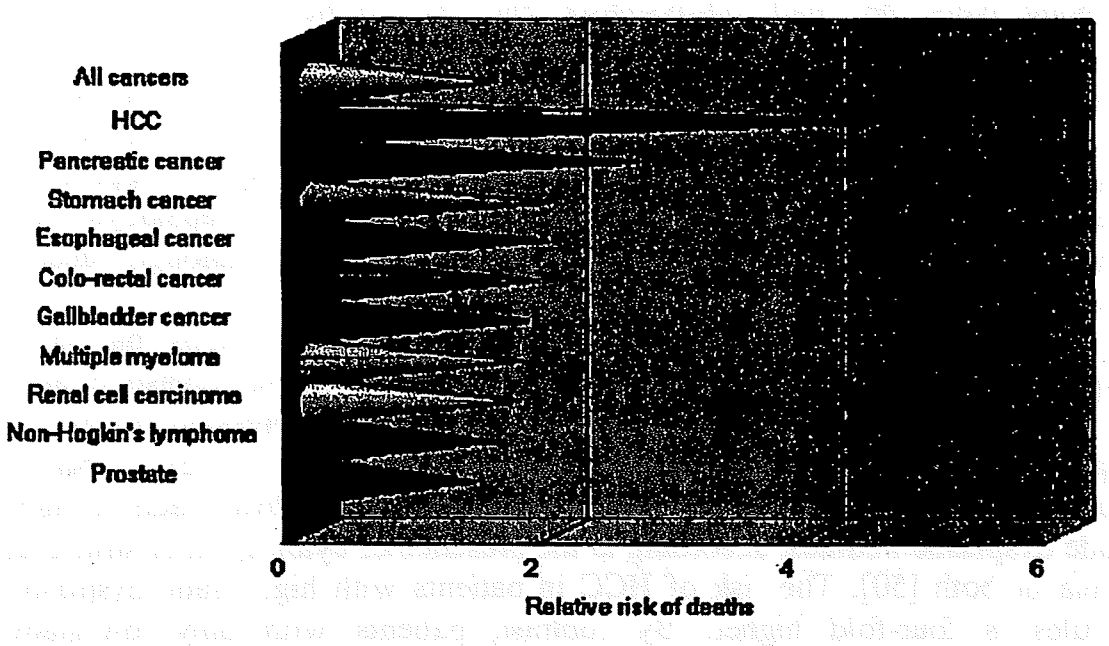

Figure 3. Relative risk of malignancies in men with BMI over 35 kg/m^2.

elevated relative risks of death from cancer, which ranged from 1.23 (95% confidence interval, 1.11 to 1.36) for death from any cancer to 4.52 (95% confidence interval, 2.94 to 6.94) for death from liver cancer. All these studies thus suggest that obesity is associated with the incidence and mortality of liver cancer.

Why obesity might be an additional risk factor for HCC? There are several reasons. Potential biologic mechanisms include increased levels of endogenous hormones (estrogens, insulin, IGF1 and leptin) associated with overweight and obesity and the contribution of abdominal obesity to fatty liver.

Obesity is associated with insulin resistance and elevated IGF, which is a mitogen that stimulates cell growth. A positive immunoreaction was found in 69% of the patients with HCC, and IGF2 was more frequently immunodetected in HCC with fatty change than without, suggesting IGF-2 may play an important role in both fatty degeneration and in the proliferation of HCC cells [54]. In addition, hepatic steatosis, frequently seen in obesity, predisposed to lipid peroxidation and excess free radical activity with the potential risk of genomic mutations. In obese patients with no history of smoking or biochemical evidence of diabetes mellitus, hypertension, hyperlipidaemia, renal or liver disease or cancer, the plasma level of malondialdehyde (MDA) is significantly higher and the activity of erythrocyte copper zinc-superoxide dismutase (CuZn-SOD) and glutathione peroxidase (GPX) is lower than in subjects with healthy BMI, suggesting that obesity is an independent risk factor for plasma lipid peroxidation and depletion of erythrocyte cytoprotectic enzymes in humans [55]. Experimental data from *ob/ob* mice that spontaneously

develop liver cancer late in life while displaying insulin resistance and fatty liver from an early age showed that fatty liver is associated with hepatocyte hyperplasia and decreased apoptosis rate, suggesting that fatty liver itself could have a permissive carcinogenic role and, therefore, represent a premalignant condition [56].

Serum leptin levels are increased significantly in patients with obesity and NAFLD, indicating that leptin resistance may exist. Leptin plays some possible roles in modulating the hepatic fibrogenic response to chronic liver injury. Leptin and its receptor (Ob-R) may play important roles in angiogenesis and metastasis in HCC [57, 58]. Furthermore, the nuclear receptor peroxisomal proliferator activated receptor (PPAR)-alpha plays a central role in sensing excess free fatty acids and up-regulating the genetic program of fatty acid disposal. On the other side, however, this protective mechanism, at least in rats, may be a predisposition to carcinogenesis [59].

Liver related morbidity and mortality increase significantly once cirrhosis develops. Autopsy series demonstrate that cirrhosis is more common in obese individuals than in those with a normal BMI. There are at least two reasons why obesity might increase the risk for cirrhosis. First, obesity is strongly associated with NAFLD, a chronic liver disease that sometimes progresses to cirrhosis (30). Second, obesity is an independent risk factor for liver fibrosis in other, common liver diseases, such as chronic HCV hepatitis and alcoholic liver disease.

8.1. Mechanism(s) for obesity-related increases in liver disease increased prevalence of NAFLD and insulin resistance

The incidence of steatosis and steatohepatitis increase with obesity. Obesity is also strongly associated with systemic resistance to insulin. Indeed, the latter is a stronger predictor of liver disease severity than the former. For example, studies in non-obese humans demonstrate that systemic insulin resistance is common in individuals with simple steatosis. It tends to be more prevalent, as well as more severe, in those with NASH and is most common and severe in patients with cirrhosis.

Systemic insulin resistance largely reflects the insulin sensitivity of skeletal muscle. Chronic, systemic resistance to insulin is strongly associated with a constellation of disorders (obesity, type 2 diabetes, hypertension, hypertriglyceridemia, low levels of high-density lipoproteins) that has been dubbed the "metabolic syndrome". Therefore, NAFLD is almost certainly a component of the metabolic (i.e., insulin resistance) syndrome. However, whether or not NAFLD is the direct result of hepatic insulin resistance (which is generally defined as an inability of insulin to suppress hepatic glucose production) has been difficult to assess in humans. Algorithims (e.g., HOMA

and QUICKI) that are derived from fasting serum insulin and glucose values, as well as glucose tolerance testing (GTT) and metabolic "clamps" provide reliable measures of systemic insulin resistance, but may overlook insulin resistance that is confined to the liver. One could consider the possibility that insulin resistance in any tissue might be restricted to a single cell type or to a particular insulin-regulated signaling cascade within an individual cell. Thus, it is virtually impossible to exclude the possibility that some degree of insulin resistance might exist in anyone with NAFLD.

The cause-effect relationship between insulin resistance and NAFLD has been easier to dissect in mice. When genetic approaches are used to target disruption of insulin signaling within murine hepatocytes, hepatocyte lipogenesis increases and steatosis ensues. Thus, at least in mice, there is no question that inducing hepatocyte insulin resistance causes hepatic steatosis. Conversely, targeted over-expression of lipoprotein lipase in murine hepatocytes increases the delivery of fatty acids to these cells. This causes steatosis and also induces hepatocyte insulin resistance. Therefore, some insults that primarily induce hepatocyte steatosis also produce secondary hepatic insulin resistance. On the other hand, because diverse factors can cause lipid to accumulate in hepatocytes, insulin resistance is certainly not the sole cause of hepatic steatosis. Whether or not other causes of hepatocyte steatosis result in hepatic insulin resistance remains unknown. There is some evidence that they may not. For example, mice with hepatic steatosis due to chronic consumption of methionine-choline deficient (MCD) diets exhibit enhanced systemic sensitivity to insulin (defined by GTT, ITT, and QUICKI). However, since the sensitivities of all insulin-regulated pathways in hepatocytes were not assessed and other liver cell populations have not been examined yet, it is not known if MCD diets induce "partial" insulin resistance within hepatocytes or "selective" insulin resistance in hepatic non-parenchymal cells.

8.2. Insulin resistance as a chronic inflammatory state

Certain biochemical/molecule parameters typify insulin resistant tissues. These include: 1) local increases in tumor necrosis factor alpha (TNF) expression, 2) activation of TNF-regulated stress-related protein kinases (e.g., Jun-N-terminal kinases (Jnk), inhibitor kappa beta kinases beta (IKKbeta) and their transcription factor targets (e.g., c-Jun, nuclear factor kappa beta (NF-κB)), and 3) induction of genes that are trans-activated by c-Jun (e.g., the protooncogene, c-jun) and NK-κB (e.g., the immunomodulatory cytokines, TNF and interleukin (IL)-6). Because "stress"-activated transcription factors interact with other transcriptional regulators (e.g., PPARs), net gene expression is ultimately dictated by which factors predominate. Variations in gene expression and stress-related kinase activities in diverse cell populations, in

turn, modulate tissue integrity and function, leading to the various end-organ consequences of insulin resistance.

A virtually identical scenario occurs during the "acute phase response" to inflammation, suggesting that insulin resistance is actually an inflammatory state. This possibility is strongly supported by evidence that C-reactive protein, a hallmark of the acute phase response, is increased in many disorders of the metabolic (insulin resistance) syndrome. Moreover, various anti-inflammatory agents improve insulin resistance by inhibiting the activity of stress-related kinases (e.g., Jnk inhibitors or aspirin, an IKK beta inhibitor) or NF-κB (e.g., TZDs) or TNF alpha (e.g., anti-TNF antibodies or adiponectin). Many of these agents (e.g., TZDs, anti-TNF antibodies, adiponectin) have already been shown to improve NAFLD in humans and/or animal models.

8.3. Obesity can alter adipokines that modulate sensitivity to insulin and inflammatory cytokines

While compelling, none of the aforementioned data prove that insulin resistance per se directly causes NAFLD. Indeed, it is plausible that insulin resistance and NAFLD are each distinct consequences of a common mediator. The recent discovery of a new adipocyte hormone, adiponectin, makes this concept particularly attractive. Adiponectin is a "good" adipokine because it: 1) antagonizes the proinflammatory factor, TNF, and 2) interacts with its cellular receptors to limit the uptake of fatty acids into hepatocytes while increasing fatty acid oxidation and lipid export from these cells. Adiponectin is also a potent insulin-sensitizing agent. Decreased levels of adiponectin have been reported in virtually every condition that leads to the metabolic syndrome, including obesity. Furthermore, improvements in the metabolic syndrome that occur in obese individuals as a result of life-style modification or TZD treatment increase adiponectin levels. In mice, adiponectin treatment also improves NASH despite persistent obesity and leptin deficiency.

Research about adiponectin is in its infancy. Hence, much remains to be learned about the factors that regulate its expression, receptors and signaling pathways in various types of cells. However, one key regulator has already been identified, i.e., TNF. This pro-inflammatory cytokine inhibits both the expression and activity of adiponectin, although the mechanisms for these actions remain uncertain.

8.4. Obesity-related factors can modulate hepatic fibrosis

Despite strong evidence supporting a causal relationship between insulin-resistant states (e.g., obesity) and fatty liver disease, the mechanisms that explain the association between obesity and cirrhosis remain obscure. As with other obesity-related diseases, it has been difficult to differentiate the effects of