tween changes in WC or BMI over a 1-year period and changes in blood pressure levels in Japanese individuals. We analyzed the data separately for each gender, because there may be gender differences in the strength of the association between various obesity parameters and blood pressure [11].

Subjects and Methods

Study Population

The study was approved by the Ethical Committees of University of Tokyo and Mitsui Memorial Hospital. Between October 2005 and October 2006, 3,312 (1,203 women, 2,109 men) individuals underwent general health screening (visit 1), and they visited our institute again in the following year (visit 2). Among these 3,312 individuals, 2,861 (1,114 women, 1,747 men) who reported not taking antihypertensive drugs at both visits were enrolled in the present study. After about 10 min of rest, systolic blood pressure (BPs) and diastolic blood pressure (BPd) were measured in the sitting position by automated sphygmomanometer, BP-203RVIII (Omron Colin, Tokyo, Japan). Blood pressure was measured twice and the mean of these data were taken. With the subject standing, WC was measured at the umbilical level to the nearest 1 cm by trained physicians and technicians [12]. After changing into a robe from our institute, height and weight were measured, and the weight of the robe was subtracted from the value indicated by the scales. Age, WC, BMI, and BPs at visit 1 were designated age1, WC1, BMI1, and BPs1, respectively. Similarly, WC, BMI, and BPs at visit 2 were designated WC2, BMI2, and BPs2, respectively. %dWC, %dBMI, and %dBPs were defined as (WC2 – WC1)/WC1 × 100, (BMI2 – BMI1)/BMI1 × 100, and (BPs2 – BPs1)/BPs1 × 100, respectively.

Laboratory Analysis

Blood samples were taken from the subjects after an overnight fast. Serum levels of total cholesterol (TC), HDL cholesterol (HDL-C), and triglycerides (TG) were determined enzymatically. Serum uric acid was measured by the uricase-peroxidase method, hemoglobin A_{1C} was determined using the latex agglutination immunoassay. Serum creatinine was measured by TBA-200FR (Toshiba Medical Systems, Tochigi, Japan) using commercially available kits, Accuras Auto CRE (Shino-test, Tokyo, Japan), according to the manufacturer's instructions. Accuracy control was performed every day by constructing X-bar and R charts using commercially available standards. Estimated glomerular filtration rate (eGFR) was calculated by the following equation: $eGFR = 194 \times (\text{serum creatinine})^{-1.094} \times (\text{age})^{-0.287}$ (× 0.739 if female) [13]. Serum insulin was measured by enzyme immunoassay. Homeostasis model assessment insulin resistance (HOMA-IR) was calculated in these individuals according to the following formula: HOMA-IR = [fasting immunoreactive insulin (μU/ml) × fasting plasma glucose (mg/dl)]/405 [14].

Statistical Analysis

Data are expressed as the mean ± SD unless stated otherwise. Analyses of variance with trend analysis, Tukey's post-hoc analysis and multiple regression analysis were conducted as appropriate to assess the statistical significance of differences between groups using computer software Dr. SPSS II (SPSS, Inc., Chicago, Ill., USA). A value of $p < 0.05$ was taken to be statistically significant.

Results

Baseline Characteristics

As described in the Methods section, among the 3,312 individuals who underwent general health screening visited our institute again in the following year; 2,861 (1,114 women, 1,747 men) who reported not taking antihypertensive drugs at both visits were enrolled in the current study (table 1). The mean ± SD of the interval between the two visits of the individuals enrolled was 355 ± 52 days. The mean ± SD age of the enrolled women (51.3 ± 9.9 years) and men (52.5 ± 10.1 years) was significantly smaller than that of the women (60.7 ± 8.3 years) and men (59.0 ± 8.5 years), respectively ($p < 0.001$), who were excluded because of the antihypertensive medication at either or both visits. Similarly, the mean BMI values of enrolled women (21.2 ± 2.9) and men (23.5 ± 2.7) were significantly smaller than those of the excluded women (22.5 ± 3.2) and men (25.0 ± 2.8), respectively ($p < 0.001$).

WC1 ranged between 51.8 and 118.5 cm, and a WC1 ≥90 cm was found in 71/1,114 women (6.4%), and a WC1 ≥85 cm was found in 183/1,114 men (16.4%). BMI1 ranged between 13.1 and 39.4. A BMI1 ≥25 was found in 110/1,114 women (9.9%) and 453/1,747 men (25.9%), and BMI1 ≥30 was found only in 12/1,114 (1.1%) women and 33/1,747 (1.9%) men. The correlation coefficients between %dWC, %dBMI, %dBPs, WC1, BMI1, and BPs1 are described in table 2. The correlation between %dWC and %dBMI was found to be moderate in men ($r = 0.476$), whereas it was weak in women ($r = 0.241$). The relationship between %dBMI and %dBPs was found to be statistically significant in the both genders. On the other hand, the relationship between %dWC and %dBPs was statistically significant only in men. Among the study subjects, it was reported that 60 subjects experienced a WC change of –10 cm or less, and 94 subjects experienced a WC change of +10 cm or more. After excluding these 154 individuals from the study population, the results obtained were not essentially changed (data not shown). It was calculated that a 10% weight gain (loss) over a 1-year period was associated with a 3.88 mm Hg BPs gain (loss) in women and a 9.86 mm Hg BPs gain (loss) in men.

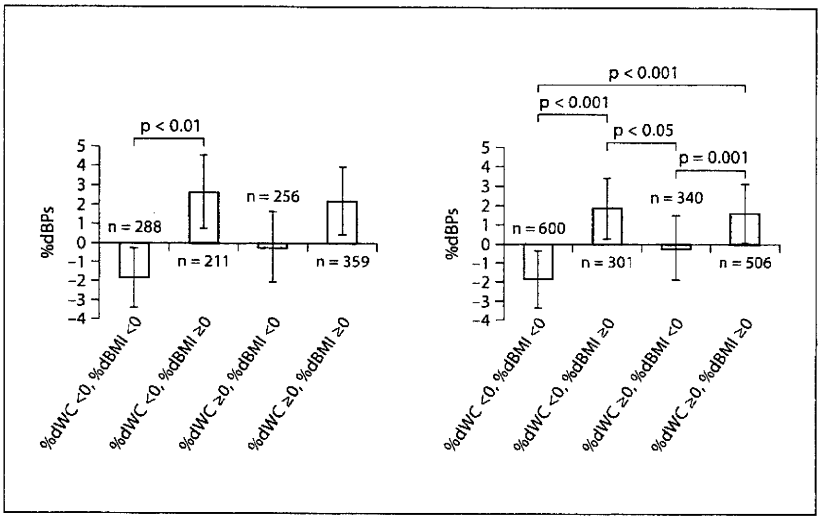

Fig. 1. Comparison of the age-adjusted %dBPs in four subgroups categorized according to the gain or loss of %dWC and %dBMI values. p values were from the result of the Tukey's post-hoc analysis following analyses of variance. Mean ± 95% confidence interval is shown in each group.

Table 1. Clinical characteristics and laboratory data at the first visit

Variables	Whole	%dBPs				p value
		first (range: −40 ~ −7)	second (range: −7 ~ 0)	third (range: +1 ~ +6)	fourth (range: +6 ~ +52)	
Number	2,861	714	809	639	699	
Women/men	1,114/1,747	288/426	314/495	251/388	261/438	0.712
Age, years	52.0 ± 10.1	52.8 ± 10.1	51.4 ± 9.9	51.8 ± 10.0	52.2 ± 10.2	0.047
Height, cm	164.8 ± 8.4	164.5 ± 8.3	165.2 ± 8.5	164.7 ± 8.5	164.7 ± 8.6	0.379
Weight, kg	61.8 ± 11.5	61.8 ± 11.4	62.0 ± 11.6	61.5 ± 11.3	61.8 ± 11.7	0.883
BMI, kg/m^2	22.6 ± 3.0	22.7 ± 3.0	22.6 ± 3.1	22.5 ± 3.0	22.6 ± 3.1	0.781
WC, cm	81.8 ± 9.1	82.0 ± 9.1	81.8 ± 9.3	81.5 ± 9.0	81.9 ± 9.0	0.851
Systolic BP, mm Hg	120.9 ± 18.0	128.7 ± 18.3	121.8 ± 17.0	118.5 ± 16.7	114.2 ± 16.8	<0.001
Diastolic BP, mm Hg	76.4 ± 11.4	79.3 ± 11.3	76.8 ± 10.9	75.5 ± 11.0	73.7 ± 11.5	<0.001
LDL cholesterol, mg/dl	129.2 ± 31.1	131.4 ± 31.5	128.3 ± 29.5	127.1 ± 30.9	130.1 ± 32.4	0.051
HDL cholesterol, mg/dl	61.2 ± 15.3	60.8 ± 15.0	61.8 ± 15.7	61.4 ± 15.6	60.7 ± 15.0	0.465
Triglyceride, mg/dl	109.9 ± 71.4	115.7 ± 69.9	104.7 ± 61.8	109.8 ± 81.0	110.1 ± 73.4	0.030
Uric acid, mg/dl	5.4 ± 1.3	5.4 ± 1.3	5.5 ± 1.3	5.4 ± 1.4	5.5 ± 1.4	0.688
Fasting glucose, mg/dl	95.2 ± 20.0	96.8 ± 20.4	95.1 ± 21.1	94.2 ± 18.0	94.7 ± 20.0	0.072
Hemoglobin A1C, %	5.3 ± 0.7	5.3 ± 0.7	5.3 ± 0.7	5.3 ± 0.7	5.3 ± 0.7	0.506
HOMA-IR	1.5 ± 1.1	1.6 ± 1.1	1.5 ± 1.1	1.4 ± 1.0	1.5 ± 1.0	0.066
Blood urea nitrogen, mg/dl	14.0 ± 3.4	13.8 ± 3.7	14.0 ± 3.2	14.2 ± 3.4	14.1 ± 3.5	0.245
Serum creatinine, mg/dl	0.8 ± 0.3	0.8 ± 0.4	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.764
Estimated glomerular filtration rate	68.6 ± 11.8	68.3 ± 11.4	69.3 ± 12.0	68.4 ± 11.8	68.1 ± 11.8	0.177
Antidiabetic medication, n (%)	51 (1.8)	12 (1.7)	20 (2.5)	10 (1.6)	9 (1.3)	0.335
Current smoker, n (%)	680 (23.8)	179 (25.0)	184 (22.7)	139 (21.8)	178 (25.5)	0.298

Data are means ± SD, unless stated otherwise. BMI = Body mass index; WC = waist circumference; HOMA-IR = homeostasis model assessment of insulin resistance. %dBPs was calculated by the following equation: (BPs at the second visit − BP1 at the second visit)/(BP1 at the second visit) × 100 (%). p value is for trend.

Table 2. Pearson's correlation coefficient of obesity indices and blood pressure parameters

	%dWC	%dBMI	%dBPs	WC1	BMI1	BPs1
Women						
%dWC						
r	–					
p value	–					
%dBMI						
r	0.241	–				
p value	<0.001	–				
%dBPs						
r	−0.014	0.097	–			
p value	0.635	0.001	–			
WC1						
r	−0.317	−0.053	−0.028	–		
p value	<0.001	0.078	0.350	–		
BMI1						
r	−0.026	−0.087	−0.029	0.787	–	
p value	0.393	0.004	0.331	<0.001	–	
BPs1						
r	−0.025	−0.055	−0.325	0.365	0.409	–
p value	0.396	0.064	<0.001	<0.001	<0.001	–
Men						
%dWC						
r	–					
p value	–					
%dBMI						
r	0.476	–				
p value	<0.001	–				
%dBPs						
r	0.116	0.232	–			
p value	<0.001	<0.001	–			
WC1						
r	−0.268	−0.089	−0.031	–		
p value	<0.001	<0.001	0.189	–		
BMI1						
r	−0.054	−0.071	−0.026	0.830	–	
p value	0.023	0.003	0.286	<0.001	–	
BPs1						
r	−0.090	−0.077	−0.327	0.308	0.322	–
p value	<0.001	0.001	<0.001	<0.001	<0.001	–

BPs = Systolic blood pressure; WC = waist circumference; BMI = body mass index. BPs at visit 1 and visit 2 were designated BPs1 and BPs2, respectively. BMI at visit 1 and visit 2 were designated BMI1 and BMI2, respectively, and WC at visit 1 and visit 2 were designated WC1 and WC2, respectively. %dBMI, %dWC, and %dBPs were calculated by the equation (BMI2 − BMI1)/BMI1 × 100 (%), (WC2 − WC1)/WC1 × 100 (%), and (BPs2 − BPs1)/BPs1 × 100 (%), respectively.

Table 3. Multiple regression analysis between %dBPs and age1, WC1, BMI1, %dWC, and %dBMI

	β	95% CI	Standardized β	p value
Women				
Model 1				
BPs1	−0.23	−0.27 to −0.20	−0.38	<0.001
Age1	0.11	0.05 to 0.18	0.10	0.001
WC1	0.11	0.03 to 0.19	0.09	0.005
%dWC	0.01	−0.06 to 0.09	0.01	0.733
Model 2				
BPs1	−0.24	−0.28 to −0.21	−0.40	<0.001
BMI1	0.47	0.25 to 0.70	0.13	<0.001
Age1	0.13	0.07 to 0.19	0.12	<0.001
%dBMI	0.34	0.15 to 0.53	0.10	0.001
Model 3				
BPs1	−0.24	−0.28 to −0.21	−0.40	<0.001
BMI1	0.65	0.28 to 1.03	0.17	0.001
Age1	0.14	0.07 to 0.20	0.13	<0.001
%dBMI	0.39	0.19 to 0.60	0.11	<0.001
WC1	−0.08	−0.21 to 0.05	−0.06	0.244
%dWC	−0.08	−0.17 to 0.01	−0.06	0.071
Men				
Model 1				
BPs1	−0.22	−0.25 to −0.19	−0.35	<0.001
WC1	0.15	0.08 to 0.22	0.11	<0.001
%dWC	0.28	0.17 to 0.39	0.11	<0.001
Age1	0.02	−0.03 to 0.07	0.02	0.467
Model 2				
BPs1	−0.22	−0.25 to −0.19	−0.35	<0.001
%dBMI	0.80	0.64 to 0.96	0.22	<0.001
BMI1	0.41	0.23 to 0.59	0.10	<0.001
Age1	0.05	0.00 to 0.10	0.05	0.035
Model 3				
BPs1	−0.22	−0.25 to −0.19	−0.35	<0.001
%dBMI	0.82	0.63 to 1.00	0.22	<0.001
BMI1	0.38	0.04 to 0.72	0.10	0.027
Age1	0.05	0.00 to 0.10	0.05	0.046
WC1	0.01	−0.11 to 0.14	0.01	0.845
%dWC	−0.03	−0.16 to 0.11	−0.01	0.705

BPs = Systolic blood pressure; WC = waist circumference; BMI = body mass index. Standardized β values are the estimates resulting from an analysis performed on variables that were standardized. BPs at visit 1 and visit 2 were designated BPs1 and BPs2, respectively. BMI at visit 1 and visit 2 were designated BMI1 and BMI2, respectively, and WC at visit 1 and visit 2 were designated WC1 and WC2, respectively. %dBMI, %dWC, and %dBPs were calculated by the equation of (BMI2 − BMI1)/BMI1 × 100 (%), (WC2 − WC1)/WC1 × 100 (%), and (BPs2 − BPs1)/BPs1 × 100 (%), respectively.

Model 1 = Independent variables include age, BPs1, WC1, and %dWC; model 2 = independent variables include age, BPs1, BMI1, and %dBMI; model 3 = independent variables include model 1 + BMI1, and %dBMI.

Multiple Linear Regression Analysis

In multiple regression analysis, in which age1, WC1, BPs1, and %dWC were used as independent variables (model 1), %dWC was found to be an independent predictive value for %dBPs in men, but not in women (table 3). In a model where age1, BMI1, BPs1, and %dBMI were used as independent variables (model 2), %dBMI was found to be an independent predictive value for %dBPs in the both genders. After including all of the age1, BPs1, WC1, BMI1, %dWC, and %dBMI in a model as independent variables (model 3), %dBMI remained to be a predictor for %dBPs in both genders. In model 3, the variance inflation factor scores of all applied independent variables were <10 (data not shown)

Comparison between Individuals with BMI Gain or Loss together with WC Gain or Loss

We then compared the %BPS values between individuals with both WC loss (%dWC <0) and BMI loss (%dBMI <0), those with both WC loss and BMI gain (%dBMI ≥0), both WC gain and BMI loss, and those with both WC gain and BMI gain during a 1-year period (fig. 1). Age-adjusted %dBPs was significantly greater in individuals with both WC loss and BMI gain compared with those with both WC loss and BMI loss. On the other hand, age-adjusted %dBPs did not significantly differ between individuals with both WC loss and BMI loss and those with WC gain and BMI loss in both genders. When the same analysis was performed after excluding 154 subjects who experienced WC change of –10 cm or less or +10 cm or more, the results obtained were not essentially changed (data not shown).

Discussion

By analyzing data from individuals who underwent general health screening for 2 consecutive years, we showed that a percent difference in BMI (%dBMI) was a statistically significant predictor for a percent difference in BPs (%dBPs) in both genders. A percent difference in WC (%dWC) was also found to be a predictor for %dBPs in men; however, it lost statistical significance after further adjustment for BMI at the first visit and %dBMI, and it was not significant in women before and after such further adjustment.

A body of evidence indicates an association between obesity parameters and blood pressure levels [15, 16]. A reduction in body weight may result in a lowering of blood pressure in overweight or obese subjects [17, 18], although the results may not be always uniform. Moore et al. [19] showed that modest weight loss over a 4-year period substantially lowered the long-term risk of hypertension in overweight adults in Framingham. Haung et al. [20] showed that weight loss occurring after 18 years of age was related to a significantly lower risk, whereas weight gain was related to greater risk of hypertension in middle-aged women. In addition, Yang et al. [21] showed that in men aged between 40 and 74 years, weight gain occurring after 20 years of age was significantly associated with prehypertension. Most of the reports studying the potential association between changes in obesity parameters and changes in blood pressure were carried over a follow-up period longer than that in the current study. Furthermore, Truesdale et al. [22] have more recently shown that weight change over a 3-year period resulted in change in blood pressure levels; men who had experienced a 10% weight gain over the previous 3 years had BPs that was 2.6 mm Hg higher. They found, however, that the impact of weight change was, albeit present, less prominent in women. Women who had experienced a 10% weight gain over the previous 3 years had BPs that was only 0.9 mm Hg higher, suggesting the presence of gender difference in the extent of association between weight change and blood pressure change. We also showed here that the magnitude of the effect of changes in obesity parameters on blood pressure changes may vary by gender (table 3).

As compared to changes in weight, and thus in BMI, fewer analyses have focused on the relationship between changes in WC and blood pressure alterations. Considering that reductions in WC have been recommended more strongly than before for the purpose of prophylaxis and/or resolution of metabolic syndrome by the government in our country [23], the impact of WC reduction (gain) in terms of alterations of atherogenic risk factors, including blood pressure and levels of glucose and lipids, is becoming a more important issue to be investigated. Therefore, we also assessed whether changes in WC were reflected by the BPs change, and whether this relationship, if present, was independent of BMI change. We found that WC change was predictive of BPs change in men but not in women. In addition, the association between %dWC and %dBPs in men lost statistical significance after controlling for BMI1 and %dBMI (table 3). In contrast, %dBMI was a predictor for %dBPs in both genders regardless of the control of %dWC, suggesting that a reduction in BMI may represent a more essential target than WC reduction in terms of blood pressure control. This concept may be further supported by our finding that mean %dBPs did

not differ significantly between individuals with %dWC <0 and those with %dWC ≥0 among individuals with %dBMI <0. In reverse, %dBPs reduction was significantly greater in individuals with %dBMI <0 than in those with %dBMI ≥0 among individuals with %dWC <0 (fig. 1).

It has been reported that, in individuals with a mean BMI of 31, change in BMI was significantly correlated with change in BPs in both genders, even after adjusting for change in waist-hip ratio [24]. In the same study, it was reported that change in waist-hip ratio was not significantly correlated with change in BPs after adjusting for BMI change in men, and that the relationship between change in waist-hip ratio and BPs change was not significant before any adjustment in women. The results of Wing et al. [24] can be said to be similar to our current observation although there is a difference between WC and waist-hip ratio.

The current study has several limitations. First, we retrospectively analyzed data on individuals who underwent general health screening at our institute for 2 consecutive years; as a result, individuals who did not visit our institute the second year for unknown reasons were not enrolled in the current study, which may cause some biases. Second, we could not specify the reasons for weight gain or loss in individuals, however, very few individuals would have been taking antiobesity medications because only one individual in each gender had a BMI of 35 kg/m^2 or more at the first visit. Third, this study population included many non-obese subjects; a BMI1 ≥30 was found only in 1.1% of women and 1.9% of men. Fourth, we excluded those subjects who were taking antihypertensive drugs at either visit. We found that BMI was significantly greater in these excluded subjects than in the study population for both genders. Lastly, although change in BMI may seem to be superior for predicting BPs change than changes in abdominal obesity, abdominal fat volume should be measured by more reliable methods, such as computed tomography, before conclusion. In addition, we have to follow the subjects for a longer period, as a recent study has shown that surrogate measures of abdominal obesity are stronger predictors of all-cause and cardiovascular death than BMI in the general population [25].

In conclusion, in individuals who underwent general health screening for consecutive years, percent change in WC was significantly associated with percent change in BPs in men, but not in women; although this association in men lost statistical significance after controlling for percent change in BMI. By contrast, percent change in BMI was significantly associated with percent change in BPs regardless of controlling for percent change in WC. Our data suggest that controlling BMI, and thus controlling body weight, may represent a more essential goal than a reduction in WC in terms of blood pressure lowering among Japanese individuals who are not taking antihypertensive medication.

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Association between metabolic syndrome and carotid atherosclerosis in individuals without diabetes based on the oral glucose tolerance test

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ABSTRACT

Introduction: Whether or not metabolic syndrome is predictive of atherosclerotic disorders may depend on the population studied. We investigated whether metabolic syndrome is associated with carotid atherosclerosis in individuals who were shown not to have diabetes mellitus based on results of the 75-g oral glucose tolerance test (OGTT).

Methods and results: Between 1994 and 2003, 3904 individuals underwent general health screening that included the OGTT. Among these 3904 individuals, 3679 had a fasting plasma glucose of <126 mg/dL (subgroup 1), and 3488 had a 2-h post-OGTT glucose value of <200 mg/dL (subgroup 2). In both subgroups, metabolic syndrome was found to be a risk factor for carotid plaque and for carotid intima-media thickening in men, and tended to be a risk factor for carotid plaque in women after adjustment for age. Among 3473 individuals who had both a fasting plasma glucose value of <126 mg/dL and a 2-h post-OGTT glucose of <200 mg/dL, 2440 did not have hypertension, which was defined as systolic and diastolic blood pressure of <140/90 mmHg and absence of use of anti-hypertensive medication. In these non-diabetic non-hypertensive individuals, the association between metabolic syndrome and carotid plaque or carotid intima-media thickening was not statistically significant even with adjustment only for age.

Conclusions: In men who did not have impaired fasting glycemia and/or in those without impaired glucose tolerance, metabolic syndrome was a predictor of carotid atherosclerosis after age adjustment, although metabolic syndrome was not found to be a predictor of carotid atherosclerosis when hypertensive individuals were excluded from the study population.

1. Introduction

Metabolic syndrome (MetS) is a cluster of metabolic and hemodynamic abnormalities linked with insulin resistance. Since components of MetS also represent risk factors for atherosclerotic disorders, it is natural that individuals with this syndrome have an increased risk for ischemic heart disease [1] and stroke [2,3]. On the other hand, the clinical utility of MetS may depend on whether the risk conveyed by this syndrome is higher than the sum of each component utilized as diagnostic criteria for MetS [4,5].

Carotid artery intima-media thickness has been reported to be a discriminator as a surrogate of cardiovascular mortality in community-dwelling Japanese people [6] and, conversely, aggregation of established major coronary risk factors has been reported to strongly influence the presence of carotid atherogenesis in the general Japanese population [7]. Previously, we reported that the presence of MetS may not increase the risk for carotid atherosclerosis in individuals without hypertension, with hypertension defined as systolic blood pressure (SBP) of ≥140 mmHg, diastolic blood pressure (DBP) of ≥90 mmHg, or the use of anti-hypertensive medication [8]. This observation suggested that the properties of MetS that present a risk for atherosclerotic diseases may differ according to the populations selected. Consistent with this idea, it was reported that MetS was not found to be associated with cardiovascular mortality in non-diabetic non-hypertensive Chinese individuals [9], and that MetS did not significantly increase the risk of mortality from cardiovascular disease in non-diabetic Mexican Americans and non-Hispanic whites [10]. In the current study, we investigated whether MetS was associated with carotid atherosclerosis in Japanese individuals who did not have diabetes mellitus based on results of the 75-g oral glucose tolerance test (OGTT).

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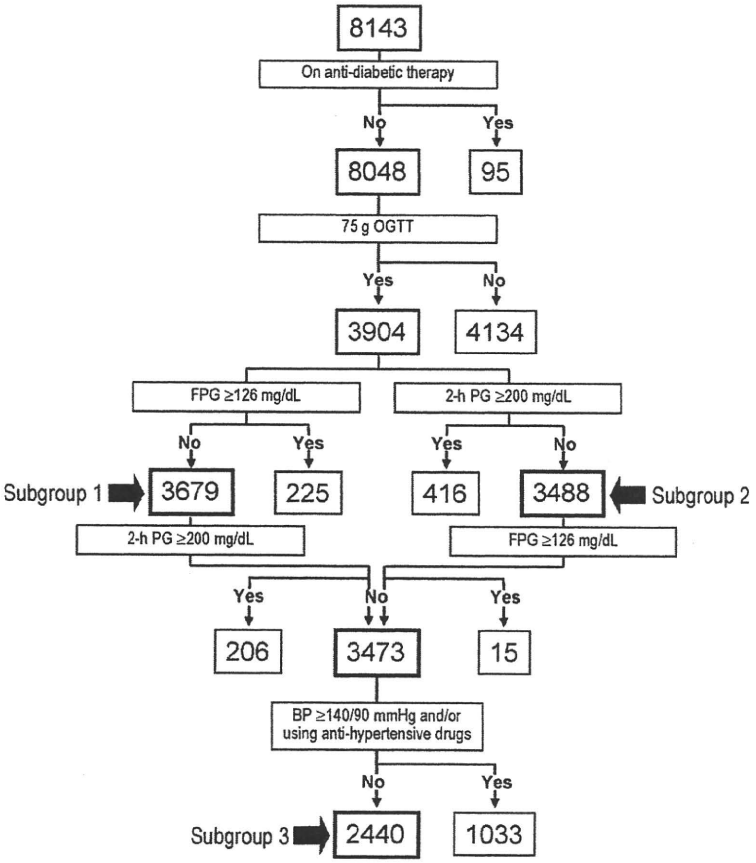

Fig. 1. Flow chart showing selection of the four subgroups.

2. Methods

2.1. Study subjects and selection of subgroups

The study was approved by The Ethical Committee of Mitsui Memorial Hospital and University of Tokyo, Faculty of Medicine. Between September 1994 and December 2003, 8143 subjects underwent general health screening including carotid ultrasonography at the Center for Multiphasic Health Testing and Services, Mitsui Memorial Hospital. Of the 8143 subjects, 95 were treated as having diabetes, and of the remaining 8048 individuals, 3904 underwent an OGTT. Among these 3904 individuals, three subgroups were sequentially selected based on various parameters (Fig. 1). Those with a fasting plasma glucose (FPG) value of <126 mg/dL were designated as subgroup 1, and those with a 2-h post-OGTT plasma glucose (2-h PG) value of <200 mg/dL were designated as subgroup 2. Subgroup 3 was comprised of subjects who met all the following conditions: FPG of <126 mg/dL, 2-h PG of <200 mg/dL, and not having hypertension. Hypertension was defined as SBP ≥140 mmHg, DBP ≥90 mmHg, or the use of anti-hypertensive medication. We also selected individuals without impaired glucose tolerance (IGT), i.e., individuals with a 2-h PG value of <140 mg/dL.

At our institute, several types of health screening programs are available, and some general health screening programs include carotid ultrasonography and/or OGTT, while others do not. However, the decision on the type of health screening was made by the individuals and/or their companies and was not decided upon or recommended by any attending physician.

2.2. Definition of MetS

MetS was defined as the presence of three or more of the following: (1) fasting glucose ≥110 mg/dL; (2) SBP/DBP ≥130/85 mmHg or taking anti-hypertensive medication; (3) triglycerides ≥150 mg/dL mmol/L; (4) HDL cholesterol <40 mg/dL in men and <50 mg/dL in women; and (5) body mass index ≥25 kg/m^2 [11].

2.3. Carotid ultrasonography

Carotid artery status was studied using high resolution B-mode ultrasonography (Sonolayer SSA270A, Toshiba, Japan) equipped with a 7.5 MHz transducer as described previously [12]. Plaque was defined to be present when there is one or more clearly isolated focal thickening(s) of the intima-media layer with thickness of ≥1.3 mm at the common or internal carotid artery or the carotid bulb. Carotid wall intima-media thickening was said to be present when intima-media thickness which was measured at the far wall of the distal 10 mm of the common carotid artery was ≥1.0 mm [12].

2.4. Statistical analysis

Logistic regression analysis was used to obtain adjusted odds ratios and their 95% confidence intervals (CIs) to predict the presence of carotid plaque or carotid intima-media thickening. Statistical analyses were carried out by using Dr. SPSS II (SPSS Inc., Chicago, IL). Results are expressed as the mean ± standard deviation (SD). A value of $p < 0.05$ was taken to be statistically significant.

Table 1
Baseline characteristics.

Variables	Subgroup 1		Subgroup 2		Subgroup 3	
	Men	Women	Men	Women	Men	Women
Number	2548	1131	2386	1102	1588	852
Age, years	58.2 ± 10.6	57.9 ± 10.4	58.0 ± 10.7	57.8 ± 10.3	56.7 ± 10.9	56.6 ± 10.5
Body mass index, kg/m^2	24.0 ± 2.8	22.2 ± 3.1	23.9 ± 2.7	22.1 ± 3.1	23.6 ± 2.6	21.7 ± 2.8
Systolic BP, mmHg	127 ± 19	121 ± 21	128 ± 19	120 ± 20	119 ± 12	123 ± 14
Diastolic BP, mmHg	79 ± 12	73 ± 12	79 ± 12	73 ± 12	73 ± 8	69 ± 9
Total cholesterol, mg/dL	206 ± 32	219 ± 35	205 ± 32	219 ± 35	205 ± 32	216 ± 35
HDL-cholesterol, mg/dL	55 ± 16	70 ± 17	55 ± 16	70 ± 17	56 ± 16	71 ± 17
Triglycerides, mg/dL	144 ± 117	96 ± 56	142 ± 98	95 ± 54	141 ± 98	95 ± 54
Uric acid, mg/dL	6.2 ± 1.2	4.7 ± 1.0	6.2 ± 1.2	4.7 ± 1.0	6.2 ± 1.2	4.6 ± 1.0
Fasting glucose, mg/dL	96 ± 10	90 ± 10	95 ± 10	90 ± 9	94 ± 9	88 ± 9
2-h OGTT glucose, mg/dL	132 ± 41	118 ± 32	125 ± 29	115 ± 26	121 ± 29	112 ± 25
Haemoglobin A1C, %	5.2 ± 0.4	5.1 ± 0.4	5.2 ± 0.4	5.1 ± 0.4	5.2 ± 0.4	5.1 ± 0.4
Hypertension, *n* (%)	863 (34)	263 (23)	788 (33)	248 (23)	0	0
Anti-hypertensive drugs, *n* (%)	336(13)	99(9)	307(13)	95(9)	0	0
Metabolic syndrome, *n* (%)	439(17)	84(7)	372(16)	72(7)	131 (8)	25(3)
Smoking status						
Never, *n* (%)	764 (30)	933 (82)	714(30)	909 (82)	465 (29)	689(81)
Former, *n* (%)	799(31)	53(5)	753 (32)	50(5)	464 (29)	44(5)
Current, *n* (%)	985 (39)	145(13)	919(39)	143(13)	659(41)	119(14)

BP indicates blood pressure, OGTT indicates oral glucose tolerance test.

3. Results

3.1. Association between MetS and carotid atherosclerosis in individuals with FPG value of <126 mg/dL (subgroup 1)

Among the 3904 individuals who underwent OGTT, 3679 (94%) had an FPG value of less than 126 mg/dL. Of these, 300 (257 men, 43 women), the FPG value was ≥110 mg/dL, thus impaired fasting glycemia (IFG), and in the remaining 3379 (2291 men, 1088 women) had an FPG value of less than 110 mg/dL (no IFG). Table 1 shows the baseline characteristics of this group according to gender. Carotid plaque was found in 823 (32%) men and 191 (17%) women and carotid intima-media thickening was found in 422 (17%) men and 122 (11%) women (Fig. 2). Age-adjusted logistic regression analysis (Model 2) showed that, in men, MetS was statistically significantly associated with carotid plaque (Table 1) and intima-media thickening (Table 2). In women, MetS tended to be associated with carotid plaque, but not with intima-media thickening after age adjustment. Similar patterns of relationships could be observed after further adjustment for total cholesterol (TC) and smoking status (Model 3). On the other hand, after full adjustment including that for components of MetS (Model 4), MetS was not significantly associated with carotid plaque or intima-media thickening in either men or women.

3.2. Association between metabolic syndrome and carotid atherosclerosis in individuals with 2-h PG value of <200 mg/dL (subgroup 2)

Among 3904 individuals who underwent OGTT, 3488 (89%) had a 2-h PG value of less than 200 mg/dL. Of these 3488 individuals 2644 (1717 men, 927 women) had a 2-h PG value of less than 140 mg/dL (no IGT) and the remaining 844 (669 men, 175 women) had a 2-h PG FPG value of ≥140 mg/dL, and thus IGT. Carotid plaque was found in 761 (32%) men and 182 (17%) women and carotid intima-media thickening was found in 378 (16%) men and 116 (11%) women. Age-adjusted logistic regression analysis (Model 2) showed that, in men, MetS was statistically significantly associated with carotid plaque (Table 2) and intima-media thickening (Table 3). In women, MetS tended to be associated with carotid plaque but not with intima-media thickening. Similar patterns of relationship could be observed after further adjustment for TC and smoking status (Model 3). On the other hand, after full adjustment that included components of MetS (Model 4), MetS was not significantly associated with carotid plaque or intima-media thickening in men or in women. There were only 15 (13 men, 2 women)

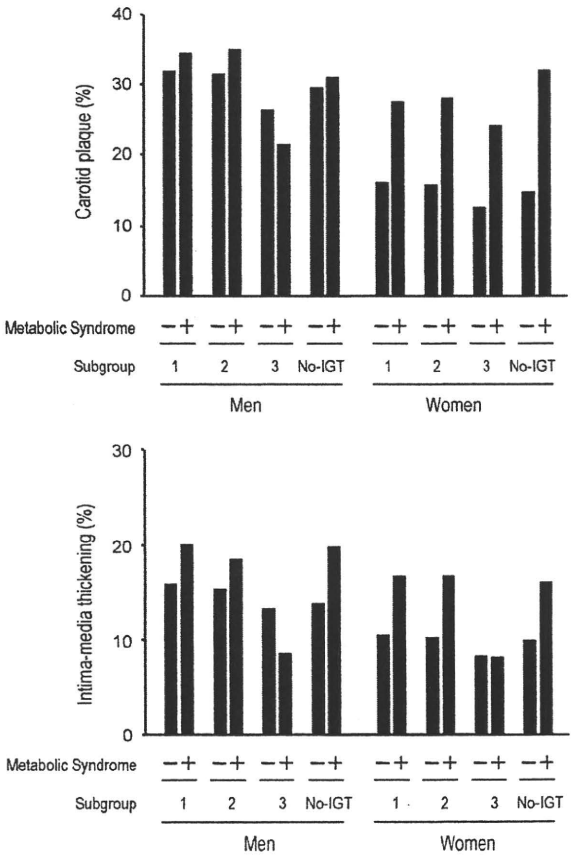

Fig. 2. Prevalence of carotid plaque and carotid intima-media thickening according to the presence or absence of metabolic syndrome in subgroups.

Table 2
Logistic regression analysis with metabolic syndrome as an independent variable and carotid plaque as a dependent variable.

Variables	Odds ratio for carotid plaque			
	Men		Women	
	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
Subgroup 1				
Model 1	1.12(0.90–1.39)	0.302	1.97(1.19–3.28)	0.009
Model 2	1.41(1.11–1.79)	0.005	1.68(0.96–2.95)	0.072
Model 3	1.30(1.03–1.67)	0.030	1.63(0.93–2.88)	0.091
Model 4	1.21(0.90–1.63)	0.209	1.61(0.79–3.29)	0.188
Subgroup 2				
Model 1	1.18(0.93–1.49)	0.170	2.06(1.20–3.55)	0.009
Model 2	1.47(1.14–1.90)	0.003	1.78(0.98–3.24)	0.058
Model 3	1.38(1.07–1.78)	0.014	1.72(0.95–3.14)	0.076
Model 4	1.23(0.90–1.69)	0.202	1.73(0.82–3.63)	0.151
Subgroup 3				
Model 1	0.77(0.50–1.19)	0.232	2.20(0.86–5.62)	0.101
Model 2	0.99(0.62–1.58)	0.971	1.89(0.66–5.43)	0.235
Model 3	0.94(0.59–1.50)	0.796	1.85(0.64–5.33)	0.254
Model 4	0.82(0.48–1.41)	0.479	2.44(0.72–8.29)	0.152

Model 1, unadjusted; Model 2, adjusted for age; Model 3, adjusted for age, total cholesterol and smoking status; Model 4, adjusted for age, body mass index, systolic blood pressure, total cholesterol, HDL cholesterol, triglycerides, fasting plasma glucose, and smoking status.

individuals among the 3488 in subgroup 2 who had an FPG value of <126 mg/dL in addition to a 2-h PG value of <200 mg/dL, and, thus, the mode of association between MetS, carotid plaque, and intima-media thickening in this subgroup was essentially the same as that observed in total population of subgroup 2.

We also investigated the association between MetS and carotid atherosclerosis in individuals without IGT. There were 2644 individuals who did not have IGT, and among them, 61 had FPG value of ≥110 mg/dL (Fig. 2, Supplementary Tables 1 and 2). The obtained results in these subgroups were similar to those in the subgroup 2; however, association between MetS and carotid intima-media thickening was statistically significant even after multivariate adjustment in women.

Table 3
Logistic regression analysis with metabolic syndrome as an independent variable and carotid intima-media thickening as a dependent variable.

Variables	Odds ratio for carotid intima-media thickening			
	Men		Women	
	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
Subgroup 1				
Model 1	1.33(1.03–1.73)	0.031	1.74(0.95–3.19)	0.074
Model 2	1.74(1.31–2.30)	<0.001	1.40(0.72–2.73)	0.324
Model 3	1.65(1.24–2.19)	<0.001	1.38(0.70–2.70)	0.349
Model 4	0.97(0.67–1.39)	0.851	0.70(0.31–1.60)	0.398
Subgroup 2				
Model 1	1.26(0.94–1.68)	0.120	1.78(0.93–3.42)	0.083
Model 2	1.63(1.20–2.22)	0.002	1.47(0.73–2.98)	0.285
Model 3	1.55(1.13–2.11)	0.006	1.44(0.71–2.93)	0.317
Model 4	1.00(0.68–1.48)	0.993	0.71(0.30–1.67)	0.435
Subgroup 3				
Model 1	0.61(0.32–1.15)	0.125	0.99(0.23–4.28)	0.985
Model 2	0.83(0.43–1.61)	0.586	0.71(0.15–3.41)	0.673
Model 3	0.77(0.40–1.50)	0.443	0.70(0.15–3.39)	0.660
Model 4	0.52(0.24–1.11)	0.092	0.56(0.05–1.45)	0.123

Model 1, unadjusted; Model 2, adjusted for age; Model 3, adjusted for age, total cholesterol and smoking status; Model 4, adjusted for age, body mass index, systolic blood pressure, total cholesterol, HDL cholesterol, triglycerides, fasting plasma glucose, and smoking status.

3.3. Association between metabolic syndrome and carotid atherosclerosis in individuals with FPG value of <126 mg/dL, 2-h PG value of <200 mg/dL, and no hypertension (subgroup 3)

Among 3904 individuals who underwent OGTT, 2440 (63%) could be assigned to subgroups 3. Their baseline characteristics according to gender are shown in Table 1. Carotid plaque was found in 409 (26%) men and 110 (13%) women and carotid intima-media thickening was found in 202 (13%) men and 69 (8%) women. Unlike subgroups 1 and 2, MetS was not significantly associated with either carotid plaque or intima-media thickening after age adjustment, or even before any adjustment in either gender (Tables 2 and 3).

4. Discussion

Here, we have assessed whether MetS is a risk factor for carotid atherosclerosis in individuals who were determined not to have diabetes mellitus based on results of OGTT. MetS was found to be associated with carotid atherosclerosis especially in men; however, when individuals with hypertension, defined as those having SBP/DBP ≥140/90 mmHg or using anti-hypertensive medication, were excluded, the presence of MetS no longer conferred excess risk when adjustments were made only for age or even when no adjustments were made.

It is known that clustering of certain metabolic abnormalities and hypertension increases the incidence of atherosclerotic diseases [13]. However, whether such clustering of atherogenic risk factors should be separately designated as MetS has been controversial. Whether MetS is independently associated with carotid atherosclerosis has been analyzed in various populations. By analyzing data on a multi-ethnic cohort of apparently healthy individuals in Canada, Paras et al. reported that although MetS was significantly associated with measures of sub-clinical carotid atherosclerosis, this association is mediated entirely through the components of MetS that have been considered as risk factors [14]. Similarly, by analyzing data on individuals recruited from a local community in Italy, Fadini et al. demonstrated that the clustering of MetS components led to a no-more-than additive increase in carotid intima-media thickness [4]. In addition, Vaidya et al. reported that MetS did not have supra-additive association with carotid intima-media thickening [15].

In our previous study that analyzed data on subjects who underwent general health screening, we found that MetS may not be associated with carotid atherosclerosis even after adjustment only for age when individuals did not have hypertension (SBP/DBP <140/90 mmHg and not using anti-hypertensive medication) [8]. In the current study, we expanded this theme to investigate whether MetS increases the risk for carotid atherosclerosis in individuals who had no or only mild (i.e., not in the diabetic range) abnormalities in glucose metabolism. We found that in individuals with FPG values of <126 mg/dL (subgroup 1) or in those with 2-h PG values of <200 mg/dL (subgroup 2), MetS was positively associated with carotid plaque after adjustment for only age (Model 2), although the relationship was only borderline positive in women. In men, the association between MetS and carotid intima-media thickening was also statistically significantly positive after adjustment for only age. These associations lost statistical significance after adjustment for TC, smoking status, and components of MetS (Model 4), suggesting that these associations may not be independent of these factors. Attention should be given to the fact that after excluding individuals with hypertension from the analysis, the association between MetS and carotid plaque or carotid intima-media thickening was no longer statistically significant even after adjustment for only age (subgroup 3), which is in agreement with our previous finding [8].

Several previous cross-sectional and longitudinal studies have investigated whether MetS increases the risk for atherosclerotic diseases in subjects without apparent impairment in glucose metabolism. A prospective population-based study of Finnish men showed that MetS was associated with higher mortality from coronary heart disease in men without impaired fasting glycemia [16]. Wilson et al. reported that MetS was associated with increased risk for cardiovascular disease in those without diabetes [17]. Leoncini et al. reported that MetS was associated with carotid atherosclerosis in non-diabetic hypertensive individuals who attended an outpatient clinic in Italy [18]. Kawamoto et al. analyzed Japanese inpatients and found that MetS increased the risk for carotid intima-media thickening in non-diabetic subjects [19]. Tzou et al. reported that the presence of MetS increased the composite of carotid intima-media thickness of ≥75th percentile of enrolled subjects in non-diabetic young adults [20]. These results support the notion that the presence of MetS will increase the risk for carotid atherosclerosis even in non-diabetic populations; however, caution should be paid in interpreting these results, as these results were not always adjusted for each component of MetS. The present results showed that MetS was associated with carotid plaque and intima-media thickening in men in subgroups 1, and 2 after adjustment for age, TC, and smoking status, although statistically significance would be lost after further adjustment for MetS components.

We found that in the absence of hypertension (subgroup 3), the association between MetS and carotid plaque or intima-media thickening was no more statistically significant after adjustment for only age, or even when no adjustments were made. These data collectively suggested that the presence or absence of hypertension, but not an abnormality in glucose metabolism, is crucial to determine whether the presence of MetS would increase the risk for carotid atherosclerosis. A recent study showed that MetS significantly increased all-cause mortality in hypertensive community-based French individuals with a hazard ratio of 1.40 (95% CI 1.13–1.74), but not in non-hypertensive individuals, during a mean follow-up period of 4.7 years [21], which was consistent with the idea of major role played by hypertension.

This study has several limitations. First, due to the cross-sectional nature of the study, we cannot determine whether there is a causal or resultant relationship between the MetS and presence of atherosclerosis. Second, among 8048 individuals who were not taking anti-diabetic medication, we excluded 4144 individuals who did not undergo OGTT. The mean age of the 3904 individuals who underwent OGTT and those 4144 who did not were significantly different (55 ± 10 years versus 58 ± 10 years, respectively, $P < 0.001$); therefore, it could be said that there had been some selection bias, though, again, the type of health screening was not decided or recommended by the physicians.

In conclusion, we showed that MetS was associated with carotid plaque and carotid intima-media thickening in non-diabetic individuals; although, this relationship did not remain statistically significant after adjustment for MetS components. In non-diabetic non-hypertensive individuals, the association between MetS and carotid plaque or carotid intima-media thickening was not statistically significant when adjustment was made for only age or even when no adjustment were made. These data collectively indicate that presence or absence of hypertension, but not an abnormality in glucose metabolism, is crucial to determine the relationship between MetS and carotid atherosclerosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2008.10.022.

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Review

Experimental models of hepatocellular carcinoma☆

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Hepatocellular carcinoma (HCC) is a common and deadly cancer whose pathogenesis is incompletely understood. Comparative genomic studies from human HCC samples have classified HCCs into different molecular subgroups; yet, the unifying feature of this tumor is its propensity to arise upon a background of inflammation and fibrosis. This review seeks to analyze the available experimental models in HCC research and to correlate data from human populations with them in order to consolidate our efforts to date, as it is increasingly clear that different models will be required to mimic different subclasses of the neoplasm. These models will be instrumental in the evaluation of compounds targeting specific molecular pathways in future preclinical studies.

***Keywords*: Liver cancer; Hepatocellular carcinoma; Mouse models; Genetically engineered mice; Cirrhosis**

1. Introduction

Hepatocellular carcinoma is one of the world's deadliest cancers, ranking third among all cancer-related mortalities. Most cases occur in Asia and sub-Saharan Africa, where viral hepatitis is endemic. The incidence is rising in the West, likely due to the increase in patients infected with hepatitis C during the latter half of the last century [1]. The liver, unique in its capacity for regeneration following injury, also gives rise to this malignancy commonly associated with the inflammatory state of advanced fibrosis, or cirrhosis. Potentially curative therapies can be offered to approximately 30% of patients, but are complicated by a high rate of recurrence [2].

Encouraging progress has been made in understanding the molecular pathogenesis of cancer [1,2]. The discoveries of the signal transduction pathways, cascades of protein–protein interactions transmitting information from the cell surface to the nucleus, and of their link to tumor biology, are particularly impressive.

Several key mouse models have been instrumental in defining the pathogenesis of HCC by introducing genetic

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Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; TKR, tyrosine kinase receptor; HBV, hepatitis B virus; TSG, tumor suppressor gene; TSP, tissue specific promoter; Tg, transgene.

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alterations into one or more aetiologic pathways that can be targeted exclusively to the liver. Moreover, these programmed manipulations can be introduced systematically, not only in this specific organ but also at defined times during development, growth and aging of the liver.

Nonetheless, substantial challenges persist in modeling liver diseases whose natural history requires a chronic inflammatory milieu. For example, infectious (hepatitis C virus), toxic (alcohol), metabolic (non-alcoholic steatohepatitis), or congenital (hemachromatosis) diseases share inflammation and fibrosis as precursors to cancer, yet none is easily mimicked in animals. There are few rodent models of HCC arising spontaneously within a background of regenerative nodules and cirrhosis, and most depend on the administration of hepatotoxic and/or carcinogenic agents to recreate the injury–fibrosis–malignancy cycle seen in chronic human liver diseases.

Comparative genomic studies in human HCC samples have begun to identify molecular subgroups with characteristic mutations, gene expression profiles and chromosomal gains and losses [3]. Moreover, since there is no single dominant molecular pathology underlying all HCCs, it is increasingly clear that different models will be required to mimic different subclasses of the neoplasm. These models will be instrumental as pre-clinical tools to evaluate compounds targeting specific molecular pathways.

With these challenges in mind, the objective of this review is to assemble and evaluate the available models of both cirrhosis and HCC, to provide a blueprint for understanding the pathogenesis of HCC and for optimizing preclinical models for drug testing.

2. Experimental models in cancer research

Although many experiments focusing on liver physiology have been conducted in rats due to their propensity for the development of fibrosis, the laboratory mouse (*Mus musculus*) is considered among the best model systems for cancer because of the availability of gene targeting methods, as well as the animal's size and breeding capacity, its lifespan of 3 years, and its physiologic and molecular similarities to human biology [4]. Significant advances have been made in modeling cancer genetics in mice, along a spectrum that ranges from simple xenograft models to more complex, genetically modified mice. Examples of each of the following are illustrated in Table 1.

2.1. Xenograft models

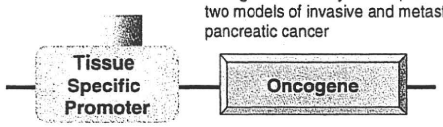
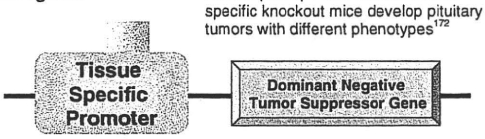
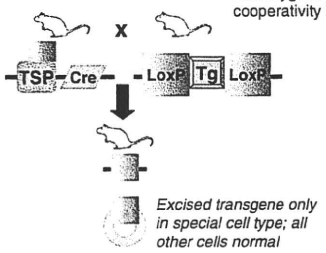
The demonstration that concentrated cancer cells grown *in vitro* could form tumors when implanted subcutaneously into an immunocompromised mouse was first established in 1969 [5]. This xenograft model has since demonstrated several advantages that explain its persistence as the mainstay of pre-clinical studies of anti-neoplastic drugs *in vivo*: the tumors are rapidly and easily induced, and their subcutaneous location enables direct measurement of tumor growth. More recently, however, several critical differences between xenograft- and patient-derived specimens have become apparent, as discussed below. In addition, cancer is now appreciated as a complex disease dependent upon the interaction between transformed cells harboring oncogenic mutations, referred to as the 'cell autonomous compartment', and their surrounding tumor environment, the 'non-cell autonomous constituents' made up of normal cells, stromal cells, and immune cells [4], features that are not part of the xenograft approach.

Mouse models of cancer were first introduced over 60 years ago. Shortly after its inception in 1955, the Developmental Therapeutics Program at the National Cancer Institute (NCI) adopted the use of three transplanted rodent models of sarcoma, carcinoma, and leukemia, for the purposes of selecting agents for clinical use in cancer patients. Thousands of molecules were tested in mice bearing murine leukemias during the first decades of modern cancer drug development, circa 1945–1969 [6]. This tumor panel was later expanded to include human tumor xenografts, with the intention to study drug activity against solid tumors [7]. In 1990, the NCI focused on the development of *in vitro* assays in 60 different cell lines in order to screen pharmaceutical agents for their potency and their selective activity against either a particular disease category or specific cell line [8,9], the most promising of which were to be subsequently evaluated in the nude mouse xenograft model.

The validity of xenografts as a predictive indicator of probable clinical activity is limited, with the most success seen in cytotoxic agents. A retrospective analysis performed by the NCI for 39 compounds in which both xenograft testing and Phase II clinical data were available showing that less than 50% of agents with activity in more than one-third of xenografts showed clinical activity ($p = 0.04$) [6]. The same study demonstrated that activity in a particular histology in a tumor model did not closely correlate with activity in the same human cancer histology [10], with the exception of non-small cell lung and ovarian cancer [11].

There are several variables inherent to the xenograft experiments which may impact on the divergent outcomes compared to human disease, including growth properties and size at initiation of treatment of xenograft tumor, ectopic versus orthotopic location of tumor, local versus metastatic disease [12], tolerance for high doses of chemotherapeutic agents in mice [13],

Table 1
Available mouse models in cancer research

	Technical Method	Advanced Mouse Models of Cancer	Current Models in HCC	Future Prospects: Wish List for HCC
Xenograft	Xenograft 	COLON, BREAST, PROSTATE: Surgical orthotopic implantation: intact fragments of human cancer, including tumors taken directly from the patient, transplanted into the corresponding organ of immunodeficient rodents[16]	Orthotopic xenograft model in which hepatoma 129 cells originating from C3H mice are injected into fibrotic livers of mice pretreated with TAA and EtOH[45]	Mouse HCC cell line derived from GEM tumor with specific molecular pathway dysregulated, with immunofluorescent marker, injected into fibrotic liver of immune-competent mice
Transgenic GEM	Constitutive Transgenic 	PANCREATIC: $Kras^{G12V}$ and chronic pancreatitis[169]; $Trp53^{R172H}$ and $Kras^{G12D}$ double transgenic driven by insulin promoter[170]; two models of invasive and metastatic pancreatic cancer	Mouse C-myc/Human E2F-1 overexpression driven by albumin promoter[171]: HCC at 6-8 months	Double transgenic overexpressing profibrotic gene combined with liver-specific oncogene
	Dominant Negative Transgenic 	PITUITARY: Rb and p27Kip1 Cdk inhibitor tissue specific knockout mice develop pituitary tumors with different phenotypes[172]	Mdr-2 knockout mice are unable to secrete phospholipids into bile, and develop cholangitis and HCC at 6-12 months[166]	Double transgenic liver-specific E-cadherin knockout and β-catenin overexpression
	Inducible Transgenic  	MELANOMA: Double transgenic combining Tet-induced overexpression of mutated $Hras^{V12G}$ and Ink4a knockout[173]	Tet-inducible Met expression under albumin promoter: 60% HCC at 12 months; tumors regressed when transgene (Tg) was inactivated[91]	Tet-induced, liver-specific overexpression of known oncogene in fibrotic mice
Endogenous GEM	Conditional Gene Targeting 	PROSTATE: Double transgenic Cre-mediated $PTEN^{-/-}$ homozygous loss and $p19Arf^{+/-}$: cooperativity in cancer development[174]	Cre-mediated liver specific PTEN-/- knockout: 66% HCC at 8 months[108]	Cre-mediated, liver-specific knockout of known tumor suppressor gene in fibrotic mice

and variability in selected endpoints. These variables can be minimized if given due consideration in the design of preclinical cancer drug experiments. However, the greatest discrepancies between success of cancer therapies in xenograft models and in human clinical trials are likely due to critical differences in both the tumor cells and their microenvironment. Natural tumor progression is a micro-evolutionary process during which increasingly aggressive clones, generated through genetic instability, emerge from an initially monoclonal lesion. Autochthonous tumors, those that evolve *in situ* from normal cells, tend to have a diminished genetic heterogeneity compared to tumor xenografts, although selective pressures of cell culture or tissue explantation can cause a rapid expansion of a certain clonal constituent of polyclonal tumors [14,15].

One solution to this disparity between cancer cell lines and human tumors is surgical orthotopic implantation, in which intact fragments of human cancer taken directly from the patient are transplanted into the corresponding organ of immunodeficient rodents, as reviewed by Hoffman [16]. This technique has been applied to breast, lung, and prostate cancer among others.

Additional advances have been made in the xenograft model through the addition of mesenchymal stem cells to weakly metastatic cancer cell lines, which enhances the ability of the cell lines to form tumors and to metastasize [17]. Wu et al. were able to isolate a side population (SP) from 29 sarcomas which preferentially formed tumors when grafted into immunodeficient mice; only cells from tumors that developed from the SP cells had the ability to initiate tumor formation upon serial transplantation [18].

Our deepening appreciation of the non-cell autonomous constituents of the tumor microenvironment, including the stroma and immune cells relevant to liver pathology in particular, provides further evidence that the xenograft model is more appropriately termed *animal culture*, as suggested by Tuveson and Frese [4].

2.2. Genetically engineered mouse models (GEM)

The most sophisticated animal models of human cancer are those that have been genetically engineered to mimic the pathophysiological and molecular features of human malignancies [4]. Such models enable the investigation of a range of discrete molecular stages that occur during tumor progression both within tumor cells and within their microenvironment; additionally, mice harboring multiple mutations provide information regarding pathway cooperativity and dependency *in vivo* [19].

Despite these strengths, there are a number of important limitations in mouse models of cancer, such as variation in basic cellular processes, as well as in telomere length and telomerase expression [20,21]. It is also well documented that identical genetic lesions can produce different pathologies in mice than in humans [22]. GEM can be categorized as either transgenic or endogenous models.

2.3. Transgenic models

Transgenic mice are those that are engineered to express either oncogenes or dominant-negative tumor suppressor genes in a non-physiologic manner due to ectopic promoter and enhancer elements [4,19]. Microinjection of recombinant DNA directly into the pronucleus of a fertilized mouse egg is the classic method for generating transgenic mice [23], but transgenic mice can also be produced through gene targeting ("knock-in") and lentiviral transduction in embryonic stem cells.

Constitutive expression of cellular and viral oncogenes and germline disruptions of tumor suppressor genes were the first approaches used to create strains of cancer-prone mice [19,24]. The cDNA constructs can contain promoter elements designed to restrict tissue tropism, so although the effect of the oncogenic gain will be constitutive, its expression can be limited to specific tissues by the use of tissue-specific promoters [19], for example the albumin promoter in liver transgenic models.

Germline tumor suppressor cell mutant mice were initially developed to parallel human inherited cancer predisposition syndromes. However, although many of these heterozygous mice were tumor-prone and demonstrated loss of the wild-type allele in their tumors, few of them developed the clinical features of the cognate human syndrome. For example, loss of the retinoblastoma gene product Rb in humans leads to retinoblastomas, osteosarcomas, and small cell lung cancer; whereas Rb heterozygote mice develop thyroid and pituitary tumors but no retinoblastomas [25]. Rb heterozygotes are able to compensate for loss of Rb, a finding that highlights the existence of shared and predictable cellular process within both species [20,26]. So, although identical genetic lesions may not perfectly recapitulate the human disease in mice, there is no doubt that these genetically engineered mice are valuable tools for understanding the underlying biological mechanisms of tumorigenesis [22]. Their ability to recapitulate the genetic features of amplified proto-oncogenes, such as c-myc [27], has contributed greatly to our understanding of cancer biology.

There are, however, additional weaknesses of these models that have spurred the development of more advanced methods. For example, because the genes affected may be vital to normal development, overexpression or ablation may lead to embryonic lethality or infertility [24]. Promoter fragments typically represent the minimal sequence required for tissue-specific expression and do not necessarily allow the same control conferred by endogenous regulatory elements [28]; for example, a typical transgene would not include all transcription factor and microRNA binding sites [4,29]. And, although the DNA fragments are thought to associate by homologous recombination before integration and in most cases insert at a single chromosome site [23], there is little control over site of integration and copy number [22]. This can result in pronounced variability of expression patterns, as the exogenous gene can affect genes near its insertion site or can be affected by endogenous control elements [22,30–32]. Also, although conventional mouse mutants may be useful for modeling familial forms of cancer, they do not mimic sporadic tumorigenesis because the

initiating mutation is present in all cells of the body, including those that constitute the tumor microenvironment [33].

2.4. Inducible systems of oncogene expression

Bujard and colleagues developed a strategy for temporally controlled and reversible transgene expression, using a tetracycline (tet-) inducible system [34]. These drug- or ligand-inducible systems involve the use of a chimeric transcriptional activator that reversibly activates a target gene in response to the administration of the inducing agent.

The *Escherichia coli* tetracycline resistance operon has been applied widely to generate cell lines and murine models with tightly regulated gene expression in response to tetracycline [35]. The tet transactivator functions either as a constitutive repressor that is inducibly inhibited by ligand to allow expression from the *tet* operon (tTA), or it acts as an inducible activator of the *tet* operon upon ligand addition (rtTA) [19]. This system has been particularly useful to study the concept of oncogene addiction; nearly all oncogenes tested thus far seem to be required not only for tumor initiation, but also for tumor maintenance [33].

2.5. Endogenous GEM: knock-out models

Endogenous GEM are those that lose the expression of tumor suppressor genes (TSG) or that express dominant-negative tumor suppressor genes or oncogenes from their native promoters [4]. The original 'knockout' mouse model entailed disruption of an allele in endogenous embryonic stem cells using a targeting vector. Biallelic disruption of TSG often results in embryonic lethality, but heterozygous mice can be used to determine the tumorigenic potential of the genes, such as the retinoblastoma tumor suppressor gene (Rb) [25]. These germline mutations are present throughout the mouse and are constitutively expressed, unlike the sporadic mutations occurring in human tumors that are surrounded by normal tissue.

2.6. Endogenous GEM: conditional gene targeting

As reviewed by Maddison et al. [22], model systems have now been developed which allow both spatial and temporal control of gene expression. These are predominantly dependent on the creation of bi-transgenic mice: those carrying a tissue-specific, inducible transactivator gene are crossed to mice carrying the allele of interest which has been engineered to be controlled by the transactivator. Offspring that carry both transgenic elements are treated with the inducer to express the transactivator gene in a specific tissue, which then acts on the desired allele. This system requires the exogenous delivery of the cre gene (usually by an adeno- or retrovirus), and the induction is irreversible.

Conditional inactivation of tumor suppressor genes relies on the ability of a viral or prokaryotic site-specific recombinase to recognize a pair of target DNA sequences and catalyze recombination at these sites, which results in either deletion or inversion of the intervening DNA sequence [19]. A commonly used tool is the Cre-Lox system, wherein Cre (*C*auses *re*combination) recombinase, isolated from bacteriophage P1, catalyses site-specific recombination between defined 34 bp Lox P sites (*Lo*cus χ of crossover *P*1) [36,37]. If gene χ is placed between two Lox P sites and then exposed to Cre, it will be excised, or 'floxed out'. An alternative system to Cre-Lox uses the FLP recombinase, which recognizes the 48 bp Frt site [38]. Transgenic mice that express recombinase from a specific promoter are bred to mice carrying conditional tumor suppressor gene mutations, so that the TSG can be bi-allelically inactivated to allow the generation of organ- and cell-lineage-specific tumors models [19].

Conditional activation of oncogenes is created by the insertion of a LoxP flanked transcriptional silencing element between the promoter and the mutant oncogene-encoding sequence. Conditional oncogenes are constructed using classic transgenic technology, but expression of the oncogene is only activated by the recombinase-mediated removal of the transcriptional silencer. This allows for tissue-specific oncogene expression [39].

This second generation of GEM, which more faithfully recapitulates sporadic tumor formation by the induction of somatic mutations in a time- and tissue-specific fashion, has provided great insight into the contribution of genes in the initiation, progression, and treatment of cancer. We will now discuss how each of these systems has been used to further our understanding of liver cancer.

3. Experimental models of hepatocellular cancer

Hepatocellular carcinoma universally arises upon a background of inflammation and fibrosis. Creation of animal models of HCC presents a particular experimental challenge because of the difficulty in modeling chronic inflammation without using carcinogens to induce liver injury, and because of the heterogeneity of molecular pathways that are dysregulated during this transition from cirrhosis to cancer.

HCC is preceded in both rodents and humans by the development of premalignant lesions including foci of altered hepatocytes and dysplastic nodules, which exhibit a higher risk of malignant evolution than normal cells [40,41]. Various genetic alterations and exposures to chemical carcinogens have been studied in animals

in order to recapitulate the phenotypic, biological, and molecular events that occur during this transformation.

3.1. Xenograft models of HCC

In a recent attempt to characterize primary human xenografts in liver cancer, seven different primary HCC cell lines were injected into SCID mice. The mice were then treated with common chemotherapeutic agents such as cisplatin and gefitinib. There were significant differences in tumor growth inhibition between xenografts, which reinforced the concern for high internal variability of this model in human cancer. Interestingly, the study concluded that most of the chemotherapeutic agents currently used in the treatment of HCC have little or no anti-neoplastic activity in these models [42].

Ma et al. have examined HCC cells expressing CD133 [43], which exhibit stem cell properties and are chemoresistant: purified CD133(+) HCC cells isolated from human HCC cell lines and harvested from xenograft mouse models survived chemotherapy in increased proportions relative to most tumor cells which lack the CD133 phenotype [44]. The inclusion of stem cell-enriched HCC cell lines will likely enhance future preclinical studies in HCC therapeutics (see Table 1).

A group of investigators at the University Hospital Bonn created an orthotopic xenograft model in which hepatoma 129 cells originating from C3H mice were injected into fibrotic livers of mice pretreated with thioacetamide by intraperitoneal injection and alcohol per oral [45]. They found that tumors in fibrotic livers grew significantly larger and more rapidly than those in normal livers, and were able to metastasize and form satellite nodules. Gene expression analysis revealed greater intratumoral expression of vascular endothelial growth factor (VEGF) and its receptor (VEGFR), and of MMP-2 and MMP-9 in the fibrotic liver tumors. This useful model provides a unique tool for testing drug efficacy in orthotopic hepatoma xenograft within the context of liver fibrosis.

3.2. Viral models of HCC

Infection causing latent or chronic viral hepatitis is the most common aetiology of HCC, comprising 80% of cases worldwide. Hepatitis B virus (HBV) is endemic in China, Southeast Asia, and sub-Saharan Africa; there, vertical transmission of the virus results in high rates of HCC. Hepatitis C (HCV) viral infection is more prevalent in the United States and Europe than either HBV or HIV [46]. The woodchuck hepatitis virus (WHV) induces a liver inflammation, injury and repair process in woodchucks similar to those of HBV-positive patients and has therefore proven to be a useful model of the disease.

3.2.1. Hepatitis B virus

HBV is a DNA virus that causes acute and chronic hepatocyte injury, inflammation, and HCC. During prolonged infection, viral DNA sequences integrate into the host cell genome, where they and the flanking cellular sequences are commonly rearranged [47], a phenomenon that can activate an adjacent cellular oncogene. In addition, viral infection can induce hepatocyte injury mediated by the antiviral cellular immune response and, to a lesser extent, by direct injury to the cells. Although most cases of HBV-associated HCC arise in a background of inflammation and fibrosis, the virus is notorious for also causing HCC in the absence of cirrhosis, most likely by integrating into the host chromosome and thereby promoting transcriptional transactivation of mitogenic factors.

The HBV virus is a circular DNA molecule containing four open reading frames encoding four HB viral proteins: preS/S, preC/C, P and X protein (HBx). The most common viral marker in HCC is the integration of HBV genomic DNA encoding HBx. In 1994, Koike et al. published their description of a transgenic mouse model demonstrating that high levels of HBx expression were sufficient to generate HCC in 84% of male transgenic mice at age 13–24 months [48] (see Table 2). Analysis of proliferation and DNA content in these mice suggested that the continued expression of HBx gene initiated tumor formation by inducing DNA synthesis and placing large numbers of hepatocytes subjective to secondary events for transformation [48]. Yu et al. also confirmed the development of HCC in HBx transgenic mice [49]. Although another research group did not see spontaneous HCC development, those HBx transgenic mice were more susceptible to chemical carcinogenesis than control mice [50]. The reason for this discrepancy is unclear, but the difference in genotype of HBV should be noted: HCC tumors arose in genotype C HBx transgenic mice but not in other genotypes [51].

Chisari et al. described a transgenic model that overproduces the hepatitis B virus large envelope polypeptide and accumulates toxic quantities of hepatitis B surface antigen (HBsAg) [52]. This hepatocellular injury initiates a programmed response within the liver, characterized by inflammation, regenerative hyperplasia, transcriptional deregulation, aneuploidy and eventually HCC. Inappropriate expression of a single structural viral gene was thereby shown to be sufficient to cause malignant transformation. The process of oncogenesis seen in this model also supports the theory that severe, prolonged cellular injury can induce a proliferative response that fosters secondary genetic events that lead to unrestrained growth [47]. However, the level of viral protein expression in this model may well surpass the expression in human infection.

Table 2
Genetically engineered models of hepatocellular carcinoma

Gene	Type of mutation or tissue promoter/construct	Phenotype (+/− and −/−)	Chemically induced/ metastasis	References
Viral models				
Hepatitis B virus large envelope protein	BgIII-A fragment of HBV encoding large envelope protein under control of albumin promoter and enhancer	Focal necrosis, inflammation, and subsequent HCC in 72% males	No metastases; rare local invasion	[47,52]
Hepatitis B virus X protein	EcoRI–BglII fragment of HBV including the X gene under its own promoter and enhancer	HCC in 84% after 13–24 months in mice with high HBx expression	Lung metastasis	[48,175,176]
Hepatitis C virus	HCV core-E1–E2 transgenic under albumin promoter and HCV core transgenic under HBV X promoter	No DEN: No HCC in either strain by 21 months. +DEN: 100% HCC at 32 weeks; HCV core-E1–E2 with largest tumors ($p = 0.008$)	DEN injected weekly × 6 weeks	[56]
Hepatitis C virus	HCV core under HBV X promoter; HCV E1–E2 under HBV X promoter	Core transgenics: 32% HCCs in male mice at 16–23 months; E1–E2 transgenics: no HCC. No evidence hepatitis	None reported	[56,59]
Hepatitis C virus	HCV core-E1–E2 transgenic under albumin promoter and the entire HCV transgenic under albumin promoter	HCC in core-E1–E2 transgenic and entire HCV transgenic after 13 months	None reported	[60]
Cell cycle models				
p53 germline knockout and liver-specific viral receptor TVA, injected with PyMT oncogene	p53 germline knockout [177] crossed with mice expressing viral receptor TVA under albumin promoter (Alb-TVA), injected at age 3 days intrahepatically with mouse polyoma virus middle T antigen	HCC in 42% of p53 null mice, in 37% of $p53^{+/-}$, and in 66% of $p53^{+/+}$ mice expressing TVA injected with PyMT at 4 months. No TVA-negative littermates developed HCC	Metastases in p53 null mice (6/16); less in $p53^{+/-}$ (1/14)	[67,177]
Trp53 and INK4a/ARF conditional mutant mice, injected with PyMT oncogene	Albumin Cre mice crossed with Trp53 conditional mutant and INK4a/ARF conditional mutant, injected at age 3 days intrahepatically with mouse polyoma virus middle T antigen	>90% HCC in combined Trp53, INK4a/ARF null mice injected with PyMT compared to single null gene	Metastases in Trp53 null mice (30%) and in combined Trp53, INK4a/ARF mice (63%) at 6 months	[68]
P53 conditional expression	Hepatoblasts transduced with oncogenic ras (Hras V12) and a tet-responsive P53 miRNA design short hairpin RNA	Complete tumor regressions when endogenous p53 reactivated in p53-deficient tumors	None reported	[69]
c-myc	c-myc over-expression under albumin enhancer/promoter [74,90,178,179]; under α1 antitrypsin promoter [180,181]	15 weeks: polyploidy cells, dysplasia >60% [179]; 15 mos: 91% adenomas [74,178]; 54% HCC [178,180,181];	None reported	[74,90,178–181]
c-myc and E2F-1	Mouse c-myc and human E2F-1 over-expression under albumin promoter	6–8 mos: 100% HCC [171,178]	None reported	[90,171,178,179]
c-myc and TGFα	c-myc over-expression under albumin enhancer/promoter; TGFα over-expression under metallothionein 1 promoter	4 mos: 70% dysplastic nodules; 18% HCC [90]	Zinc in H_2O accelerated nodule formation by 6–8 weeks	[90,182]

(*continued on next page*)

Table 2 (*continued*)

Gene	Type of mutation or tissue promoter/construct	Phenotype (+/− and −/−)	Chemically induced/ metastasis	References
SV40 T-antigen conditional and inducible expression	SV40 T-antigen expression under albumin enhancer/ promoter [74]; under major urinary protein enhancer/ promoter [183]; under metallothionein 1 promoter [184]; under α1 antitrypsin promoter [185]; under antithrombin III promoter [186]; tetracycline-inducible expression: mice expressing tTa under albumin promoter crossed with mice expressing T antigen under tTa promoter [75]	3–7 mos: adenomas and HCC [74]; 10–12 weeks: HCC[185]; after 4–6 weeks: 100% HCC [186]	Lung metastases [186]	[74,75,181,183–186]
E2F-1	E2F-1 over-expression under control of albumin enhancer/ promoter	10 mos: 100% adenomas and dysplastic nodules; 12 mos: 33% HCC	None reported	[71,90,179]
Telomere dysfunction models				
mTERT$^{-/-}$ and p53$^{+/-}$ or WT	Germline mTERT and p53 knockout over several generations and CCl_4 liver injury	50 weeks: 100% HCC in p53$^{+/-}$ both generations (G0 and G3/ G4); 44% in wild-type G0 versus 9% HCC in wild-type G3/G4	CCl_4 by IP injection 3×/ week × 4 months	[66]
Pathway specific models				
Wnt/β-catenin				
Activating mutation in β-catenin: truncated NH_2 terminal transgenic	EAB/9K/Δ N131 β-catenin construct under control of liver-specific enhancer of aldorase B gene (expressed throughout embryonic and post-natal development)	Death at 3 weeks from hepatomegaly; no dysplastic foci in liver	N/A	[127]
Activating mutation in β-catenin: exon 3 conditional knockout	Catnb$^{lox(ex3)}$ knockout and fatty acid binding protein Fabpl-cre transgenic	Death at 5 weeks from liver damage/mitochondrial swelling. No dysplastic foci in liver; +intestinal polyps	N/A	[128]
Activating mutation in β-catenin: exon 3 conditional knockout	Catnb$^{lox(ex3)}$ knockout injected with recombinant adenovirus expressing Cre from human CMV promoter	High multiplicity injection (10^9 pfu/mouse): death at 3 weeks with hepatomegaly/ mitochondrial swelling. Low multiplicity injection (10^{7-8} pfu/ mouse): No dysplastic foci in liver >6 mos	N/A	[128]
β-catenin exon 3 knockout and activated H-ras (H-ras^{G12V}) double-transgenic conditional	Catnb$^{lox(ex3)}$ knockout and H-ras (Tg$^{lox(pA)H\text{-}ras^*}$) double-transgenic with recombinant adenovirus expressing Cre from human CMV promoter	Low multiplicity infection (10^8 pfu/mouse): 100% HCC at 6 months	Intrahepatic invasion	[131]
APC knockout liver-specific	Apc$^{\Delta ex14}$ knockout (−/−) injected in tails with recombinant adenovirus expressing Cre (injections infected primarily and massively the liver)	High multiplicity infection (10^9 pfu/mouse): Death within 2 months and hepatomegaly. Lower multiplicity infection (0.5×10^9 pfu/mouse): 67% HCC at 9 months. Apc$^{+/-}$ had no liver abnormalities	None reported	[130]
β-catenin wild-type	β-catenin over-expression under control of albumin enhancer/promoter	Hepatomegaly (15% increased liver/body weight ratio); no dysplastic nodules at 24 months	N/A	[129]

Table 2 (*continued*)

Gene	Type of mutation or tissue promoter/construct	Phenotype (+/− and −/−)	Chemically induced/ metastasis	References
PI3K/Akt pathway				
PTEN$^{-/-}$	Albumin cre/PTEN$^{lox/lox}$	Steatohepatitis; Adenomas at 44 weeks and 66% HCC at 78 weeks [108]; HCC in 66% of males at 44 weeks and in 83% of males and 50% of females at 78 weeks [109]	Lung metastases	[108,109]
Insulin growth factor pathway				
IGF2 transgenic	IGF2 over-expression under control of urinary protein promoter	HCC in <10% at 18–24 months; also lymphomas, sarcomas, and thyroid carcinomas	None reported	[187]
IGF2 knockout and TGFα transgenic	TGFα over-expression under metallothionein 1 promoter [86] crossed with IGF2 heterozygous knockout mice (paternal null allele; maternal wild-type, normally imprinted)	(1) IGF2$^{wt/wt}$: no HCC; 4% adenoma; (2) IGF2$^{+/-}$: dwarves, normal liver phenotype; (3) TGFα × IGF2$^{wt/wt}$ and (4) TGFα + IGF2$^{wt/-}$: 100% HCC at 18 months	None reported. Zinc in drinking water starting at age 10 months	[188]
Epidermal growth factor pathway				
EGF transgenic	Double-transgenic of the liver construct Alb-DS4 that encodes autocrine growth factor IgEGF crossed with AAT-myc mice	EGF transgenic (Alb-DS4): mortality from HCC by age 7.1 months; EGF/myc double-transgenic: accelerated mortality to 4.4 months		[115]
Ras signaling				
H-ras	Mutant c-H-ras over-expression under albumin promoter	Hepatomegaly, lung tumors [74]	None reported	[74]
HGF/c-Met and TGF-α				
HGF transgenic	Mouse HGF expression driven by metallothionein promoter [95]; by albumin promoter[189]	Hepatomegaly; >17 months: adenomas and rare HCCs [95]; rapid recovery after partial hepatectomy, no dysplasia [189]	Most animals not given zinc because transgene expression adequate	[95,189]
HGF over-expression +/− β-catenin conditional knockout	Hydrodynamic injection of plasmid containing HGF under CMV promoter (pCMV-HGF) into wild-type and into AFP-enhancer albumin promoter-Cre floxed β-catenin knockout mice	HGF over-expression: hepatomegaly and increased Wnt/β-catenin signaling; no dysplastic nodules. HGF over-expression in β-catenin knockout: no alterations in liver	N/A	[96]
HGF + c-myc	Double-transgenic mouse c-myc driven by albumin promoter/enhancer and human HGF driven by albumin regulatory elements	Inhibition of hepatocarcinogenesis by HGF in c-myc transgenic mice: 0% HCC in HGF/c-myc versus 60% HCC at 16 months in c-myc single transgenic, even with addition of phenobarbital	Phenobarbital	[97]
HGF + TGF-α	Double-transgenic mouse TGFα over-expression under metallothionein 1 promoter and human HGF driven by albumin promoter	Increased proliferation and c-myc expression in HGF over-expressing mice. Diminished hepatocarcinogenesis by HGF in TGFα transgenic mice: 33% (3/9 mice) HCC in HGF/ TGFα versus 60% (6/10) in TGFα single transgenic	None reported	[98]
Met transgenic	Tetracycline-inducible expressing human Met under liver-specific promoter crossed with mice expressing tetracycline transactivator under liver-specific liver activating protein (MET-TRE/LAP-tTA) [91,99]	12 months: 60% HCC; tumors regressed when transgene was inactivated [91]; by 4 months, adenomas and HCC [99]. +Recurrence of HCC in mice whose original tumors had regressed on Doxycycline	None reported	[91,99]

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