2-Amino-3-Methylimidazo[4,5-f]Quinoline (IQ) Promotes Mouse Hepatocarcinogenesis by Activating Transforming Growth Factor-β and Wnt/β-Catenin Signaling Pathways

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The purposes of the present study were to investigate the modifying effects of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), a genotoxic carcinogen produced during cooking of protein-rich foods, and elucidate underlying mechanisms in a two-stage hepatocarcinogenesis mice model. Six-week-old B6C3F1 mice were subjected to two-thirds partial hepatectomy at the beginning of the study, followed by an intraperitoneal injection of diethylaminoethylsulfonate on day 1. Starting 1 week later, they were fed diets containing IQ at doses of 30, 100, or 300 ppm for 39 weeks. A dose-dependent trend for increase in eosinophilic altered foci as well as significant elevation in the incidence of hepatocellular carcinomas in the 100- and 300-ppm IQ groups as compared with initiation control group. Furthermore, IQ elevated the protein expression levels of Wnt1, transforming growth factor-β (TGF-β), TGF-β receptors 1 and 2 (TβR1 and TβR2), and phosphorylated c-Jun (p-c-Jun), while suppressing those of E-cadherin and p21WAF1/Cip1. Moreover, translocation of β-catenin to the nuclei as well as upregulated nuclear expression of c-Myc and cyclin D1, which are downstream targets of β-catenin and p-c-Jun, were detected at 100 and 300 ppm. These findings suggest that IQ exerts dose-dependent promoting effects on mice hepatocarcinogenesis by activating TGF-β and Wnt/β-catenin signaling pathways and inhibiting cell adhesion.

Key Words: IQ; hepatocarcinogenesis; TGF-β pathway; Wnt/β-catenin pathway; E-cadherin.

The incidence of hepatocellular carcinomas (HCCs) has significantly increased over the past decades, attribute to the increased prevalence of chronic liver hepatitis, especially chronic hepatitis B and C, whereas some other factors, such as dietary carcinogens, also contribute to the risk of certain types of cancer (Glade, 1999). Epidemiological studies have found a positive correlation between dietary intake of foodstuffs containing traces of heterocyclic amines and the likelihood of developing cancer (Layton et al., 1995). In the human diet, consumption of 400 g of cooked lean meat could result in exposure to several micrograms of mutagenic heterocyclic amines (Lakshmi et al., 2008). These amines can be detected in urine and their excretion as unchanged forms is increased by cytochrome P450 (P450) inhibition (Turesky et al., 2002), indicating absorption from cooked foods and P450 metabolism. 2-Amino-3-methylimidazo[4,5-f]quinoline (IQ), one of the genotoxic and carcinogenic heterocyclic amines formed by high-temperature cooking of proteinaceous food, targets multiple organs in rodents. For example, long-term treatment (675 days) with 300 ppm IQ has been shown to induce tumors in the liver, lung, and forestomach of CDF1 mice (Ohgaki et al., 1986).

The mutagenicity and carcinogenicity of IQ are considered initially to involve oxidation of the exocyclic amino group (G) to its corresponding N-hydroxy-IQ by liver CYP1A1 and 1A2 (Hammons et al., 1997). Subsequently, O-acetylation or sulfonation of the exocyclic group is believed to result in the formation of the ultimate genotoxic species, which are capable of binding to DNA, leading to formation of DNA adducts, and in turn mutations and neoplastic transformation (Lakshmi et al., 2008; Turesky et al., 2002). It has also been reported that IQ is associated with p53 gene mutations in HCCs in the cynomolgus monkey. It was further suggested that these mutations might be induced by the formation of DNA adducts of IQ in the p53 gene (Fujimoto et al., 1994). Moreover, IQ may suppress the activity of protein kinase C and block kinase activation of nuclear factor-κB and AP-1 with IL-2 gene expression in murine spleen cells (Lee et al., 1998). However, despite the fact that daily exposure to IQ and other heterocyclic amines in food may present a potent carcinogenic risk to humans, the mechanisms underlying IQ carcinogenicity are still not fully understood.

In recent years, transforming growth factor-beta (TGF-β), E-cadherin adhesion complexes, and the Wnt/β-catenin pathways have become hot topics in HCCs studies (Marijon et al., 2011; Matsuzaki et al., 2007; Polakis, 2000; Sekimoto et al., 2007; Wolfe et al., 2011). TGF-β and its isoforms initiate a
signaling cascade, which is closely linked to liver fibrosis, cirrhosis, and subsequent progression to HCCs, thus playing a unique role in its molecular pathogenesis (Giannelli et al., 2011). However, it is not clear whether there is any relationship between IQ and TGF-β or its isoforms during hepatocarcinogenesis.

β-Catenin is an E-cadherin-binding protein involved in cell-cell adhesion (Hirohashi, 1998), but it also functions as a transcriptional activator in the Wnt pathway when assembled in the nucleus with members of the T lymphocyte-specific transcription factor/lymphoid enhancer-binding factor family of binding proteins (Korinek et al., 1998). It may also be a target of IQ, which can induce β-catenin mutations in rat colon tumors, which feature elevated expression of c-Myc and c-Jun proteins (Blum et al., 2001). Whether IQ exerts effects on β-catenin and related factors in hepatocarcinogenesis remains to be clarified.

As the metabolism of IQ in the mouse is similar to human (Lakshmi et al., 2008), in the present study, we chose a two-stage mouse model to investigate the effects of different doses of IQ on hepatocarcinogenesis, focusing on underlying mechanisms involving the above-mentioned pathways.

MATERIALS AND METHODS

Chemicals and diets. IQ was purchased from the Nard Institute (Osaka, Japan), with a purity of 99.9%, and diethylnitrosamine (DEN) from Tokyo Chemical Industry Company (Tokyo, Japan). Basal diet (powdered MF; Oriental Yeast Co., Tokyo, Japan) was prepared once a month by Oriental Yeast Co. The diets containing IQ were made once a month using a roller (Universal Ball Mill UB32; Yamato Scientific Co., Tokyo, Japan) and draft chamber (GHD-1500; Oriental Giken Inc., Tokyo, Japan) set in the animal center.

Animals and procedures. One hundred and forty-five 5-week-old male B6C3F1 mice (Charles River, Shizuoka, Japan) were housed in plastic cages (five animals per cage) in an animal facility maintained under standard conditions (room temperature, 23 ± 1°C; relative humidity, 44 ± 5%; and light/dark cycle, 12 h) and were given free access to powdered diet (MF; Oriental Yeast Co.) and tap water. The animals were acclimatized for 1 week prior to the beginning of the experiment, which was conducted after obtaining approval of the Animal Care and Use Committee of Osaka City University Medical School.

The experimental protocol is shown in Figure 1. To enhance hepatocellular proliferation, two-thirds partial hepatectomy (PH) was performed in 105 mice, under anesthesia as previously described (Nicou et al., 2001). Whether IQ exerts effects on β-catenin and related factors in hepatocarcinogenesis remains to be clarified.

Histopathology. After formalin fixation, livers were embedded in paraffin, sectioned at 3-μm thickness, and stained with hematoxylin and cosin (H&E) for histopathological examination. Liver altered foci, hepatocellular adenomas, and HCCs were counted under the light microscope and categorized based on tumor size, thickness of hepatic plates, and presence of mitotic figures, according to the diagnostic criteria provided by Maronpot et al. (1999).

Immunohistochemistry. Immunohistochemical analyses were performed on liver tumors, hepatocellular adenomas (HCAs) and HCCs, of mice in PH→DEN groups as described previously (Kakehashi et al., 2010). In this study, we employed primary antibodies against β-catenin (dilution 1:100, 6B3; Cell Signaling Technology, Beverly, New York), TGF-β (dilution 1:500, ab66043; Abcam Inc., Cambridge, MA), E-cadherin (dilution 1:500, ab53033; Abcam), Wnt1 (dilution 1:200, ab15251; Abcam), and c-Myc (dilution 1:100, ab39688; Abcam). After testing the different antigen-retrieval methods and negative controls, immunohistochemical procedures were optimized.

Extraction of nuclear proteins and whole cell proteins. Three tumors in each group of animals that underwent PH followed by DEN injection were used for protein expression analysis. Nuclear protein mixtures were prepared with a Nuclear Extraction Kit (Panomics, Santa Clara, CA) according to the manufacturer’s instructions. Whole cell proteins of the tumors were also extracted as described previously (Kang et al., 2008).

Western blotting analysis. Supernatants obtained from mice liver tumors (20 μg protein per sample) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes. After blocking for 1 h in 5% milk-Tris-buffered solution-Tween solution, membranes were incubated with primary antibodies at 4°C overnight, followed by the appropriate secondary antibody for 1 h at room temperature. Bands were visualized using the ECL PLUS system (Amersham, U.K.). Primary antibodies against β-catenin (dilution 1:2000, 6B3; Cell Signaling Technology), TGF-β (dilution 1:1000, ab66043; Abcam Inc.), E-cadherin (dilution 1:2000, ab53033; Abcam), TGF-β receptor 1 (TjR1, dilution 1:1000, ab31013; Abcam) and TGF-β receptor 2 (TjR2, dilution 1:1000, ab53168; Abcam), Wnt1 (dilution 1:500, ab15251; Abcam), c-Myc (dilution 1:1000, ab39688; Abcam), p21 (dilution 1:1000, ab7960; Abcam), cyclin D1 (dilution 1:1000, sc-8396; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), p53 (dilution 1:2000, sc-6243; Santa Cruz), phosphorylated c-Jun (p-c-Jun, dilution 1:1000, sc-16312; Santa Cruz), β-actin (dilution 1:10000, ab49900; Abcam), and histone H2A (acidic patch, dilution 1:2000, 07-146; Upstate Biotechnology, Billerica, MA) were applied.

FIG. 1. Experimental design for the two-stage mouse hepatocarcinogenesis model. The numbers of mice from groups 1 to 8 were 20, 20, 20, 15, 10, 20, 20, and 20, respectively.

**TABLE 1.** Schedule of experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>0.24 hour</th>
<th>1 week</th>
<th>40 weeks</th>
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<tr>
<td>1</td>
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<td>8</td>
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Densitometric analysis was carried out using TINA software (Raytest, Straubenhardt, Germany) with β-actin used as a whole cell internal control and histone H2A as a nuclear internal control. Data of relative integrated density values of selected bands are shown as bar charts.

**Statistical analysis.** All mean values were expressed as means ± SDs. Statistical analyses were performed using the Statlight program (Yukms Co., Ltd, Tokyo, Japan). Incidences of liver lesions were compared using the Chi-squared test. Homogeneity of variance was tested by the Bartlett test in PH/DEN groups. Differences in mean values between the control and IQ-treatment groups were evaluated by the two-tailed Dunnett’s test when variance was homogeneous and the two-tailed Steel’s test when variance was heterogeneous. Homogeneity of variance was tested by the F-test in groups undergoing PH without DEN initiation and groups without PH. Differences in mean values between the control and IQ-treatment groups were evaluated by the two-tailed Student’s t-test when variance was homogeneous and the two-tailed Aspin-Welch’s t-test when variance was heterogeneous. P values less than 0.05 were considered significant.

**RESULTS**

**Body and Liver Weights, Water Intake, Food Consumption, and IQ Intakes**

The results for body and liver weights, water intake, food consumption, and IQ intakes of mice are presented in Table 1. Eighteen animals in the initiation control and IQ-treated groups died during the study. Three mice each in G1 and G2, one mouse in G3, and two mice in G4 died from the hepatectomy operation during the first week after the start of the experiment. The causes of death of the other seven mice found dead are unclear, one mouse in G5 at week 3, one mouse each at weeks 15 and 19 in G1, one mouse in G2 at week 24, one mouse in G7 at week 25, and one mouse each at weeks 33 and 37 in G6. One mouse each in G3 and G4 died from liver extensive hemorrhage induced by tumors at weeks 36 and 38, respectively. As the first tumor was observed at week 36, all mice surviving until this week were included in the final analysis. IQ did not affect average food consumption and water intake, but suppressed body weight gain, mostly in the group administered the dose of 300 ppm.

In the groups undergoing PH followed by DEN initiation, final body weights of 300-ppm IQ-treated animals were significantly decreased compared with the DEN control group. No significant changes in final body weights were observed in the 30- and 100-ppm IQ groups. Similarly, final body weights of mice subjected to PH without DEN initiation followed by IQ administration at a dose of 300 ppm were significantly lower than that in the vehicle control group. Moreover, in the groups without PH, significant decrease of final body weight was observed at 300 ppm.

No significant changes in absolute and relative liver weights were observed in mice receiving IQ after PH with DEN initiation compared with the DEN control group. Furthermore, no significant changes of liver weights were found in animals subjected to PH without DEN initiation followed by 300 ppm IQ. In groups without PH, significant decrease of absolute but not relative liver weight was noted in the 300-ppm IQ group compared with the DEN control group.

**Histopathological Evaluation**

The results of the histopathological analysis are shown in Table 2. In mice undergoing PH with DEN initiation, 100- and 300-ppm IQ treatments significantly increased the development of HCCs, whereas in animals subjected to PH without DEN injection, no significant promoting effect of IQ was found. Thus, in the PH/DEN groups, incidence and multiplicity of HCCs with 100-ppm (33%, 0.33 ± 0.49) and 300-ppm (50%, 0.83 ± 1.19) IQ groups were significantly elevated as compared with the DEN control group (0%, 0 ± 0). In mice receiving 300 ppm IQ, multiplicity of total tumors (3.50 ± 2.11) was also significantly elevated compared with the initiation control group (1.73 ± 2.15). Furthermore, in the PH/DEN groups,
the rates for eosinophilic altered foci (control: 11.1%, 30 ppm IQ: 28.2%, 100 ppm IQ: 30.6%, 300 ppm IQ: 47.6%) and eosinophilic HCCs (control: 7.7%, 30 ppm IQ: 37.5%, 100 ppm IQ: 40.4%, 300 ppm IQ: 44.1%) showed a trend for dose-dependent increase, whereas the rates for basophilic altered foci and HCAs showed a decreasing trend (data not shown). Mixed type altered foci were not influenced by IQ application. No tumors were observed in animals not subjected to PH.

**Effects of IQ on Protein Expression of E-Cadherin as Well as Components of TGF-β and Wnt/β-Catenin Signaling Pathways Found by Immunohistochemistry**

In the liver tumors of all IQ-administered mice in PH→DEN groups, E-cadherin expression, a cell surface transmembrane glycoprotein with a key role not only in intracellular adhesion but also in cell differentiation, was significantly suppressed. Furthermore, in IQ-administered groups, E-cadherin was localized in the cytoplasm, whereas in the DEN initiation group, it was generally detected on the cell surfaces (Fig. 2a–d).

Protein expression of TGF-β, a known key regulator in HCCs development, was found to be elevated as compared with DEN control group tumors in all IQ-treatment groups (Fig. 2e–h).

In line with the findings for TGF-β, rise in protein expression of Wnt1, particularly in 300-ppm IQ-treated mice, but also present in 30- and 100-ppm IQ groups, was found as compared with the initiation control group (Fig. 2i–l).

β-Catenin membranous localization was observed in the liver tumors of DEN initiation control animals, whereas in IQ-treated mice, a shift to cytoplasmic and/or nuclear expression was found, most evident in the 100- and 300-ppm IQ-treatment groups (Fig. 2m–p). In parallel, c-Myc overexpression in the nuclei of tumor cells was also detected in mice administered IQ at 100 or 300 ppm (Fig. 2q–t).

**TABLE 2**

<table>
<thead>
<tr>
<th>Groups (number of group and effective mice)</th>
<th>PH → DEN (G1, 15)</th>
<th>30 ppm IQ (G2, 16)</th>
<th>100 ppm IQ (G3, 19)</th>
<th>300 ppm IQ (G4, 13)</th>
<th>PH → (G5, 9)</th>
<th>300 ppm IQ (G6, 19)</th>
<th>DEN → (G7, 19)</th>
<th>300 ppm IQ (G8, 20)</th>
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<tr>
<td>Incidence of lesions (%)</td>
<td></td>
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<tr>
<td>HCAs</td>
<td>12 (80)</td>
<td>13 (81.3)</td>
<td>14 (73.7)</td>
<td>12 (92.3)</td>
<td>0 (0)</td>
<td>2 (10.5)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>HCCs</td>
<td>0 (0)</td>
<td>4 (25)</td>
<td>6 (33.3)*</td>
<td>6 (50)**</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total tumors</td>
<td>12 (80)</td>
<td>13 (81.3)</td>
<td>14 (73.7)</td>
<td>13 (100)</td>
<td>0 (0)</td>
<td>2 (10.5)</td>
<td>0 (0)</td>
<td>0 (0)</td>
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<tr>
<td>Multiplicity of lesions</td>
<td></td>
<td></td>
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<tr>
<td>HCAs</td>
<td>1.73 ± 2.15</td>
<td>2.38 ± 2.59</td>
<td>2.47 ± 2.63</td>
<td>2.61 ± 1.33</td>
<td>0 ± 0</td>
<td>0.21 ± 0.63</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
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<tr>
<td>HCCs</td>
<td>0 ± 0</td>
<td>0.31 ± 0.6</td>
<td>0.33 ± 0.49*</td>
<td>0.83 ± 1.19**</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
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<tr>
<td>Total tumors</td>
<td>1.73 ± 2.15</td>
<td>2.69 ± 2.67</td>
<td>2.80 ± 2.93</td>
<td>3.45 ± 1.97*</td>
<td>0 ± 0</td>
<td>0.21 ± 0.63</td>
<td>0 ± 0</td>
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*Note. G, group.

*p < 0.05; **p < 0.01 versus DEN control group.

Alteration in Protein Expression in Liver Tumors of Mice Detected by Western Blotting

Pronounced upregulation of TGF-β protein expression was found in the liver tumors of mice treated with IQ at doses of 100 ppm (1.45-fold) or 300 ppm (1.75-fold, Figure 3A). Furthermore, compared with the initiation control group, expressions of TβR1 and TβR2 were also enhanced by IQ, particularly at 300 ppm (2.01- and 2.10-fold, respectively). Expression of p-c-Jun, which can be stimulated by TGF-β, was also significantly increased in animals treated with 300 ppm IQ (1.57-fold, Fig. 3A). As with TGF-β and its receptors, expression of Wnt1 was also significantly elevated in 100-(1.95-fold) and 300-ppm (2.56-fold) IQ groups (Fig. 3A).

Similar to the results of immunohistochemical examination, expression of E-cadherin was significantly decreased in all IQ-treated animals, with the fold of 0.33, 0.22, and 0.24 from 30 to 300 ppm group, respectively, compared with the initiation control group (Fig. 3A).

Intriguingly, in the tumors of mice treated with 30–300 ppm IQ, a parabola-shaped change in protein expression levels of p21WAF1/Cip1 was found. That is, compared with DEN control group, no change of p21WAF1/Cip1 expression was observed in both high-dose IQ groups, whereas in the 30-ppm group, overexpression (1.37-fold) was clear (Fig. 3A).

Expression of p53 in the 300-ppm IQ-treatment group (2.56-fold) was significantly higher than in of initiation control group (Fig. 3A).

In the nuclei of liver tumor cells in mice administered 100 and 300 ppm IQ, overexpression of β-catenin (1.42- and 1.44-fold, respectively) was obvious compared with animals administered DEN alone (Fig. 3B), apparently coordinated with elevation of Wnt1 and suppression of E-cadherin. Expression of c-Myc (1.36- and 1.37-fold, respectively) and cyclin D1 (1.77- and 1.79-fold, respectively), the downstream of p-c-Jun and β-catenin, were also markedly increased by
treatment with IQ at doses of 100 and 300 ppm when compared with the initiation control group (Fig. 3B).

**DISCUSSION**

This study provided the first evidence of promoting effects of 100 and 300 ppm IQ on liver carcinogenesis in male B6C3F1 mice. No significant influence of IQ compared with the PH alone control group was detected when mice were not initiated with DEN. In groups without PH, regardless of DEN injection and IQ treatment, no tumors were observed. However, in mice undergoing PH followed by DEN initiation, dose-dependent promotion effects of IQ on development of HCCs were clearly demonstrated. In this two-stage mouse model, not only DEN injection but also PH was necessary to detect the modifying effects of IQ on mice hepatocarcinogenesis. The progression step of hepatocarcinogenesis is irreversible and can be enhanced by administration of a “progressor,” namely, a genotoxic compound (Dragan et al., 1994). In rat liver carcinogenesis, most foci of altered hepatocytes, which finally progress to HCCs, are eosinophilic/clear cell foci (di Francesco et al., 2007). In line with these data, administration of IQ, a genotoxic compound, dose-dependently increased eosinophilic altered foci and HCAs in the PH → DEN groups in this study. The augmentation of such lesions could clearly contribute to progression to HCCs.

Previous study demonstrated that administration of IQ alone, for almost 2 years, could straightforwardly increase liver tumor

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**FIG. 2.** Immunohistochemistry for E-cadherin (a–d), TGF-β (e–h), Wnt1 (i–l), β-catenin (m–p), and c-Myc (q–t) in the livers of mice. In the DEN control group (a), E-cadherin was located on the surfaces of tumor cells generally (arrow), whereas in IQ-treatment groups (b–d), it was decreased (arrow) and the localization was mostly cytoplasmic (arrow). Overexpression of TGF-β was detected in tumors of mice treated with IQ (e–h) compared with DEN alone (e). Wnt1 positivity in mouse liver tumors was also increased by IQ treatment (i–l), most prominently at 300 ppm (l) compared with the initiation control (i). β-Catenin showed membranous distribution in liver tumors of DEN control animals (m, arrow), but a shift to cytoplasmic/nucleus expression was observed in tumors of IQ-treatment groups, particularly in the 100- and 300-ppm IQ groups (o and p, arrow). Compared with the DEN control case (q), the numbers of c-Myc-positive nuclei were increased by IQ in the 100- and 300-ppm IQ groups (s and t, arrow). Bars represent 20 μm.
FIG. 3. Western blotting analysis of TGF-β, TβR1, TβR2, p-c-Jun, E-cadherin, Wnt1, p21[wp1/Cip1], p53, β-catenin, c-Myc, and cyclin D1 protein expression in liver tumors of mice. (A) Compared with DEN initiation control group, significant overexpression of TGF-β (1.45- and 1.75-fold, respectively) and TβR1 (1.53- and 2.01-fold, respectively) was detected in the 100- and 300-ppm IQ groups. Protein expression of TβR2 (2.10-fold) and p-c-Jun (1.57-fold) was significantly increased only at 300 ppm. Significantly decreased expression levels of E-cadherin were observed in all IQ-treated animals, with the fold of 0.33, 0.22, and 0.24 from the 30-, 100-, and 300-ppm group, respectively, compared with the initiation control group. Expression levels of Wnt1 were significantly higher in IQ-administered groups, especially in the high-dose groups (2.56-fold). Significant overexpression of p21[wp1/Cip1] was detected in the 30-ppm IQ group (1.37-fold), whereas no changes were found at the 100- and 300-ppm IQ doses. Increased expression of p53 was detected in mice treated with IQ at a dose of 300 ppm (2.56-fold). β-Actin was used as an internal control. (B) Compared with DEN initiation control group, overexpression of nuclear β-catenin (1.42- and 1.44-fold, respectively), c-Myc (1.36- and 1.37-fold, respectively), and cyclin D1 (1.77- and 1.79-fold, respectively) was detected in mice-administered IQ at doses of 100 and 300 ppm. Histone H2A was used as a nucleus internal control. Significant compared with the DEN alone group (*p < 0.05, **p < 0.01). IDV, integrated density value.
incidence in CDF1 mice and Fisher 344 (F344) rats (Ongaki et al., 1986). However, the underlying mechanisms of IQ effects have not been fully understood. Furthermore, because Lakshmi et al. (2008) pointed out that mouse IQ metabolism was similar to that for human but different from that for rat and the metabolism could influence tumorigenicity, the parameters affecting IQ tumorigenicity in rat and mouse may be different. In our study, considering similarity in the metabolism and the cost-effectiveness, instead of F344 rats, a two-stage 40-week mouse model was applied.

Naugler et al. (2007) proposed that estrogen could reduce liver cancer risk in females by using mouse DEN model, in which tumor appears in 100% of males but only in 13% females. In view of their findings, male Balb/c3F1 mice were chosen in our experiment because DEN was used as liver tumor initiation carcinogen after PH. Moreover, F344 male rats (68%) administered with IQ for almost 2 years showed higher incidence of HCCs than that of female (45%), although male CDF1 mice (41%) had lower incidence of liver tumors than that of female (75%, Ongaki et al., 1986). Furthermore, P450 enzymes, which were responsible for the occurrence of sex differences in rat liver microsomes, appear rather specifically depending on the sex hormones (Kamataki et al., 1983). Considering the species variation and gender bias, sex-dependent effects induced by IQ need further investigation.

In the report by the World Health Organization of International Agency for Research on Cancer (1993), from 1 kg of cooked meats and fish, 0.02–158 μg of IQ was detected. Although the dosages used in the present experiment are several orders of magnitude apart from human consumption levels, daily exposure may present a potent carcinogenic risk to humans.

To elucidate the underlying mechanisms of IQ promoting effects in PH and DEN-treated animals, we focused on alterations in protein expression of elements participating in TGF-β and Wnt/β-catenin signaling. Emerging evidence indicates that TGF-β participates in oncogenic processes, such as growth stimulation and increased motility (Wakefield and Roberts, 2002). On the molecular level, binding of TGF-β to TβR2 leads to recruitment of TβR1. The resultant complex then triggers the downstream cascade to regulate gene transcription (Moustakas and Heldin, 2002). In the present study, dose-dependent increases of protein expression levels of TGF-β, TβR1, and TβR2 were observed in liver tumors of IQ-administered animals, thus indicating that IQ at high doses of 100 and 300 ppm activated the TGF-β signaling pathway. Moreover, eosinophilia may be caused by proliferation of mitochondria in rat hepatocytes (Reznik-Schulier and Gregg, 1983). Interestingly, protein TGF-β localizes in mitochondria (Heine et al., 1991). Therefore, it is reasonable to speculate that enhanced expression of TGF-β might be related to the eosinophilic changes in IQ-treated groups in this study.

It has been previously reported that expression of E-cadherin, a cell surface transmembrane glycoprotein with a key role not only in intracellular adhesion but also in cell polarity, growth, and differentiation, is negatively regulated by TGF-β (Cano et al., 2000). Dissociation of the E-cadherin adhesion complex is considered a prerequisite for tumor cell invasion and metastasis formation (Hirohashi, 1998; Wolfe et al., 2011). Furthermore, loss of or reduced membrane positivity of E-cadherin has been described in HCCs (Wu et al., 2011). In general, E-cadherin staining is strong in well-differentiated cancers that maintain their cell adhesiveness but is reduced in poorly differentiated tumors, which have lost cell-cell adhesion and show strong invasive behavior (Wijnhoven et al., 2000). Here, we demonstrated significant inhibition of E-cadherin protein expression in mouse liver tumors by IQ at doses of 100 and 300 ppm, indicating that IQ treatment might enhance the malignant potential of tumor cells after DEN initiation.

Cooperation of the TGF-β and Wnt/β-catenin signaling pathways in carcinogenesis has been described (Takaku et al., 1998). Thus, progression of benign adenomatous polyps to invasive forms of carcinoma is accelerated when members of both the TGF-β and the Wnt signaling cascades are altered. β-Catenin is an essential component of both intercellular junctions and Wnt signaling (Polakis, 2000). An increased expression of Wnt1 and nuclear accumulation of β-catenin have been recognized as features of an activated Wnt signaling pathway (Willert and Jones, 2006). In addition, a multivariate analysis earlier demonstrated poorer prognosis and higher rate of tumor recurrence in patients with nuclear accumulation of β-catenin (Inagawa et al., 2002). Our finding of β-catenin translocation from plasma membranes to the cytoplasm and nucleus, associated with increased levels of Wnt1, c-Myc, and cyclin D1 expression in the 100- and 300-ppm IQ groups, points toward a novel mechanism of the promotion effect of IQ on HCCs development. In view of these results, we speculate that with Wnt/b-catenin signal activation, β-catenin targets c-Myc and cyclin D1, which leads to their upregulation, due to the influence of IQ.

TGF-β is capable of stimulating c-Jun NH2-terminal kinase (JNK) activity (Hocevar et al., 1999) and its activation has been reported to induce TβR1/p21WAF1/Cip1 as well as JNK/c-Myc, thus resulting in either inhibition or promotion of rat hepatocarcinogenesis (Matsuzaki et al., 2007; Sekimoto et al., 2007). Here, we could demonstrate increased protein expression of p21WAF1/Cip1 in the liver tumors of mice by 30 ppm IQ but no change with either the 100-ppm or the 300-ppm dosage. Furthermore, p21WAF1/Cip1 suppression coordinated with overexpression of p-c-Jun was observed in tumors of the 100- and 300-ppm IQ-treatment groups. In line with our previous results, significant elevation of p21WAF1/Cip1 was demonstrated with a low dose of IQ (10 ppm), even below that required for IQ-mediated carcinogenic effects in the liver of rats (Wei et al., 2011). Moreover, suppression of p21WAF1/Cip1 expression by 100- and 300-ppm doses of IQ might be suggested to be a result of an inhibitory effect on significantly upregulated c-Myc at these doses.
Expression of p21^WAF1/Cip1 can also be controlled by p53 (Wu et al., 2003), for which overexpression has been found in many types of human malignancies. There is evidence supporting high levels of p53 alteration in HCCs (Guzman et al., 2005) and significantly elevated expression was detected in the 300-ppm IQ group in the present study. The findings indicate that IQ affects the malignant progression of liver tumors with high levels of p53.

Because increased TGF-β and Wnt/β-catenin signaling have previously been associated with liver carcinogenesis (Polakis, 2000; Sekimoto et al., 2007), the possibilities cannot be ruled out that latency-to-tumor is reduced after exposure to IQ and that tumors would have arisen anyway in the DEN initiation group after 2 years and that these tumors would have had the increased TGF-β and Wnt/β-catenin signaling. Nevertheless, in the present study, increased expressions of proteins TGF-β, TβR1, TβR2, Wnt1, p-c-Jun, p53 as well as nuclear proteins β-catenin, c-Myc, and cyclin D1 are at least partly responsible for dose-dependent promotion effects of IQ on mouse hepatocarcinogenesis when compared with these proteins expression profiles in the DEN initiation control group.

In conclusion, in the present study, IQ dose-dependently exerted promotion effects on mouse hepatocarcinogenesis. In this two-stage mouse model, the mechanism of IQ promotion effects on formation of HCCs appears likely to be related to the simultaneous stimulation of TGF-β and Wnt/β-catenin signaling pathways in hepatic tumors (Fig. 4). Increase of expression levels of TGF-β complex preponderantly stimulates JNK (p-c-Jun) and simultaneously suppressed expression of E-cadherin. The latter could have contributed to translocation of β-catenin from cell membranes to the cytoplasm and/or nucleus. Furthermore, increased expression of Wnt1 also might activate β-catenin and promote its translocation. Finally, induction of the downstream targets c-Myc and cyclin D1 occurred, resulting in the promotion of mouse hepatocarcinogenesis. Increased c-Myc may repress p21^WAF1/Cip1 expression, which indirectly suppresses cancer inhibition.

FIG. 4. Proposed model for IQ promotion mechanisms with regard to mouse hepatocarcinogenesis. IQ simultaneously stimulates TGF-β and Wnt signaling pathways in the two-stage mouse model. Increase in TGF-β complexes preponderantly stimulates JNK (p-c-Jun) and synchronously suppresses expression of E-cadherin. Subsequently, loss of E-cadherin expression results in translocation of β-catenin from cell surface to the cytoplasm and/or nucleus. At the same time, Wnt1 activates β-catenin and promotes its translocation. Thereafter, activation of downstream targets of β-catenin and TGF-β/JNK/c-Myc cascade, c-Myc and cyclin D1, results in promotion of HCCs development. Increased c-Myc may repress p21^WAF1/Cip1 expression, which indirectly suppresses cancer inhibition.

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