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 mouse 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 diethylnitrosamine 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 the incidence of hepatocellular carcinomas was observed, along with 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 food-stuffs 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-hydroxyl-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 blockade activation of nuclear factor-kappaB 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 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). Briefly, after ventral laparotomy, the left lateral, left median, and right median lobes were ligated and excised. Twenty-four hours after the PH, 105 mice were randomly divided into six groups. Seventy-five mice and right median lobes were used from groups 1 to 8 were 20, 20, 20, 15, 10, 20, and 20, respectively.

Histopathology. After formalin fixation, livers were embedded in paraffin, sectioned at 3-μm thickness, and stained with hematoxylin and eosin (H&E) for histopathological examination. Liver altered foci, hepatic 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.

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, and 20, respectively.

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 (TβR1, dilution 1:1000, ab31013; Abcam) and TGF-β receptor 2 (TβR2, dilution 1:1000, ab35168; Abcam), Wnt1 (dilution 1:500, ab15251; Abcam), c-Myc (dilution 1:1000, ab39688; Abcam), p21WAF1/Cip1 (dilution 1:1000, ab7960; Abcam), and histone H2A (acidic patch, dilution 1:2000, 07-146; Upstate Biotechnology, Billerica, MA) were applied.

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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-treated animals were 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,
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 p21<sup>WAF1/Cip1</sup> was found. That is, compared with DEN control group, no change of p21<sup>WAF1/Cip1</sup> 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).
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
FIG. 3. Western blotting analysis of TGF-β, TβR1, TβR2, p-c-Jun, E-cadherin, Wnt1, p21 WAF1/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 WAF1/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 (Ohgaki 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 B6C3F1 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%, Ohgaki 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-Schuller 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/β-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.
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 stimulated 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.

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