Original Article

Red meat intake may increase the risk of colon cancer in Japanese, a population with relatively low red meat consumption

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在一個紅肉攝取相對較低民族日本，攝取較多紅肉會增加結腸癌罹患風險

亞洲人口飲食從傳統飲食轉變為西式飲食，紅肉的攝取量也隨之增加。然而，一些前瞻性研究已證實，這些人的結腸直腸癌的發展最可能受到攝取紅肉的不良影響。我們在一個日本的大型世代研究，評估男女性在紅肉及加工肉品的攝取與特定部位結腸直腸癌的罹患風險之間的相關性。在 1995-1998 年間，80,658 位 45-74 歲男女性填寫了一份經過效度測試食物頻率問卷。至 2006 年底，共追蹤了 758,116 人年，有 1,145 個結腸直腸癌病例被診斷。紅肉攝取較高的女性參與者，其罹患結腸癌的風險顯著較高[攝取最多的五分之一比起最低的五分之一，其複迴歸危害比(95%信賴區間)：1.48 (1.01, 2.17; 趨勢 p=0.03)]；男性則是總肉類攝取較高者，其風險也顯著較高[危害比=1.44 (1.06, 1.98; 趨勢 p=0.07)]。就特定部位而言，女性在近端結腸癌具有正相關，而男性則為遠端結腸。加工肉品的攝取與罹患結腸或直腸癌的風險皆不具相關性。總之，以西方的標準而言，中年日本人攝取紅肉最多的五分之一的量僅算中等量，但是已經足以增加罹患風險。

關鍵字:肉、結腸癌、直腸癌、前瞻性研究、日本

Genome-Wide Association Study in East Asians Identifies Novel Susceptibility Loci for Breast Cancer

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Abstract

Genetic factors play an important role in the etiology of both sporadic and familial breast cancer. We aimed to discover novel genetic susceptibility loci for breast cancer. We conducted a four-stage genome-wide association study (GWAS) in 19,091 cases and 20,606 controls of East-Asian descent including Chinese, Korean, and Japanese women. After analyzing 690,947 SNPs in 2,918 cases and 2,324 controls, we evaluated 5,365 SNPs for replication in 3,972 cases and 3,852 controls. Ninety-four SNPs were further evaluated in 5,203 cases and 5,138 controls, and finally the top 22 SNPs were investigated in up to 17,423 additional subjects (7,489 cases and 9,934 controls). SNP rs9485372, near the TGF-β activated kinase (*TAB2*) gene in chromosome 6q25.1, showed a consistent association with breast cancer risk across all four stages, with a *P*-value of 3.8×10^{-12} in the combined analysis of all samples. Adjusted odds ratios (95% confidence intervals) were 0.89 (0.85–0.94) and 0.80 (0.75–0.86) for the A/G and A/A genotypes, respectively, compared with the genotype G/G. SNP rs9383951 ($P=1.9\times10^{-6}$ from the combined analysis of all samples), located in intron 5 of the *ESR1* gene, and SNP rs7107217 ($P=4.6\times10^{-7}$), located at 11q24.3, also showed a consistent association in each of the four stages. This study provides strong evidence for a novel breast cancer susceptibility locus represented by rs9485372, near the *TAB2* gene (6q25.1), and identifies two possible susceptibility loci located in the *ESR1* gene and 11q24.3, respectively.

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Introduction

Breast cancer is one of the most common malignancies diagnosed among women worldwide, including those living in East Asian countries. Genetic factors play an important role in the etiology of both sporadic and familial breast cancer [1]. In the past two decades, more than 1,000 reports have been published addressing the association between variants in candidate genes and breast cancer risk. However, only a few genetic risk factors have been confirmed for this common malignancy [2]. Recent genome-wide association studies (GWAS) have identified approximately 20 common genetic susceptibility loci for breast cancer [3–14]. However, these newly-identified genetic factors, along with known high-penetrance breast cancer susceptibility genes explain less than 30% of the heritability for this cancer [2,15]. Furthermore, most GWAS were conducted among women of European ancestry, and many of the variants discovered in European-ancestry populations showed only a weak or no association with breast cancer in other ethnic groups [16,17]. For example, only 8 of 12 breast cancer risk SNPs identified in women of European ancestry were directly replicated in Chinese population [18]. Therefore, GWAS conducted in non-European women are needed to fully uncover the genetic basis for breast cancer susceptibility. Herein, we report results from a large GWAS of breast cancer conducted in East Asian women.

Results

A total of 19,091 female breast cancer cases and 20,606 female controls—including 23,891 Chinese, 11,907 Korean and 3,809 Japanese women—were included in the present study (Table 1). In Stage I, we analyzed 690,947 SNPs in 2,918 breast cancer cases and 2,324 community controls recruited from studies conducted in Shanghai, China (Figure 1, Text S1). Top 5,365 SNPs were investigated in Stage IIa including 1,613 Chinese cases and 1,800 Chinese controls recruited from studies conducted in Shanghai, China. Of the SNPs evaluated, 68 SNPs showed an association with breast cancer risk at $P \leq 0.05$ with the same direction as observed in Stage I. We performed a meta-analysis for the remaining 4,913 SNPs with data available from both Stage IIa and Stage IIb (2,359 Korean cases and 2,052 Korean controls). Twenty-six SNPs showed an association with breast cancer risk with $P_{meta} \leq 0.05$ and the association was consistent among Stages I, IIa and IIb. These SNPs, along with the 68 SNPs mentioned above, were selected for Stage III replication in 4,712 cases and 4,496 controls. Finally, based on the results of the first three stages, 22 top SNPs were selected for Stage IV evaluation in 7,489 cases and 9,934 controls.

SNP rs9485372 showed a statistically significant association with breast cancer risk in each of the four stages (Table 2). The OR (95% CI) per A allele was 0.88 (0.81–0.95), 0.86 (0.81–0.92), 0.94 (0.88–1.00) and 0.90 (0.85–0.94), respectively, for stages I to IV. The association with this SNP was remarkably consistent across all but one small study (Figure 2A). Pooled analysis of samples from all studies produced OR (95% CI) of 0.90 (0.87–0.92) and *P*-value of 3.8×10^{-12}, which is substantially lower than the conventional genome-wide significance level of 5×10^{-8} based on conservative Bonferroni adjustment of multiple comparisons at $\alpha = 0.05$, providing strong evidence for an association of this SNP with breast cancer risk.

Two other SNPs, rs9383951 and rs7107217, were also consistently replicated in each of the three replication sets. The C allele of rs9383951 was associated with decreased risk with OR (95% CI) of 0.82 (0.73–0.93), 0.90 (0.81–1.00), 0.91 (0.82–1.00), and 0.88 (0.81–0.96), respectively, for stages I to IV (Table 2). The P-value reached 1.9×10^{-6} in the pooled analysis of samples from all four stages. For SNP rs7107217, the ORs (95% CI) per C allele were 1.13 (1.04–1.23), 1.11 (1.04–1.18), 1.07 (1.00–1.14) and 1.05 (1.01–1.10), respectively, for stages I to IV, respectively (Table 2). Analyses with all subjects combined showed OR (95% CI) of 1.08 (1.05–1.11) and *P* value of 4.6×10^{-7}. Again, the association of breast cancer risk with these two SNPs was very consistent across the vast majority of participating studies (Figure 2B and 2C).

Stratified analyses showed that the associations with these three SNPs were consistent in all three East Asian populations, although the association for SNPs rs9485372 and rs7107217 was not significant for Japanese subjects, probably due to a small sample size (Table 3). Associations of these three SNPs with breast cancer risk were similar when stratified by menopausal or estrogen receptor status and none of the heterogeneity tests was statistically significant (Table S1). No significant interaction was observed with other risk factors (Table S1). After adjusted for the top 5 or 10 principal components, the results did not change significantly (Table S2).

Both SNPs rs9485372 and rs9383951 are located at chromosome 6q25.1, approximately 2.34 Mb and 350 kb from the SNP rs2046210 that we previously reported for breast cancer risk [8]. None of these three SNPs, however, are in LD ($r^2<0.1$) in any of the three populations (Asian, European and Africans) as determined using data generated in the HapMap or any of the study populations included in the current study (Table S3 and Figure S1). In an analysis including all 30,153 subjects who were genotyped for three SNPs in 6q25.1, all three SNPs remained strongly associated with breast cancer risk after mutual adjustment of the other 2 SNPs with P values of 1.4×10^{-12}, 1.3×10^{-4}, and 6.0×10^{-39} for SNPs

Author Summary

Breast cancer is one of the most common malignancies among women worldwide. Genetic factors play an important role in the etiology of breast cancer. To identify common genetic susceptibility alleles for breast cancer, we performed a four-stage genome-wide association study in 19,091 cases and 20,606 controls among East-Asian women. Single nucleotide polymorphism (SNP) rs9485372, near the TGF-beta activated kinase 1 (*TAB2*) gene at chromosome 6q25.1, was associated with breast cancer risk ($P=3.8\times10^{-12}$). SNPs rs9383951, located in intron 5 of the estrogen receptor 1 (*ESR1*) gene, and rs7107217, located at 11q24.3, were also consistently associated with breast cancer risk in all four stages with a combined *P* of 1.9×10^{-6} and 4.6×10^{-7}, respectively. This study provides strong evidence for a novel breast cancer susceptibility locus represented by rs9485372, near the *TAB2* gene (6q25.1), and identifies two possible susceptibility loci located in the *ESR1* gene and 11q24.3, respectively.

rs9485372, rs9383951 and rs2046210, respectively (Table S4). No significant interaction was observed for these three SNPs (Table S5). We also created a genetic risk score (GRS) to evaluate the combined effect of three SNPs located in 6q25.1 (Table S6). Compared with women carrying 0–1 risk variants, women carrying 6 variants had over two-fold increased risk with an OR (95% CI) of 2.36 (1.89–2.96) and a P value of 1.3×10^{-47}.

A total of 376 SNPs were successfully imputed in the LD blocks including rs2046210 and rs9485372 and the whole *ESR1* gene with RSQ≥0.3 and minor allele frequency (MAF)≥0.05. Among them, 27 SNPs showed an association with breast cancer risk with P≤0.05 after adjusted for age, rs9485372, rs9383951 and rs2046210 (Table S7). With the exception of rs4591859 and rs7776340 in the locus of rs2046210 and rs7768330 in the locus of rs9383921, all other SNPs are in the same LD block within the *ESR1* gene (Figure S2). No additional SNP in the rs9485372 locus showed an association with breast cancer risk at $p<0.05$ after adjusted for rs9485372, rs2046210, and rs9383921.

Discussion

In this large GWAS conducted in East-Asian women including 19,091 cases and 20,606 controls, we provided strong evidence for a novel breast cancer susceptibility locus represented by rs9485372 and suggestive evidence for two other loci, represented by SNPs rs9383951 and rs7107217.

We previously reported a genetic susceptibility locus at 6q25.1, represented by rs2046210, for breast cancer risk [8]. The newly identified SNPs, rs9485372 and rs9383951, also are located at chromosome 6q25.1. However, these three SNPs are not in LD and are thus representing independent breast cancer susceptibility loci. All of them were associated with breast cancer risk after mutual adjustment of the other two SNPs. SNP rs9485372 is approximately 31 Kb upstream of the TGF-β activated kinase 1/MAP3K7 binding protein 2 (*TAB2*) gene (Figure 3). The protein encoded by this gene is an activator of MAP3K7/TAK1, which is required for the IL-1 induced activation of NF-κB and MAPK8/JNK. The TGF-β pathway plays a major role in breast cancer development and progression [19]. The MAP kinases pathway is critical in regulating cell growth and cell death [20] and may

Table 1. Selected characteristics of studies participating in the Asia Breast Cancer Consortium.

Study Stage[a]	Ethnicity	No. of cases	No. of controls	age[b]	Menopause (%)[c]	ER+ (%)
Stage I						
Shanghai-I	Chinese	2,918	2,324	51.7/50.3[d]	42.9/41.7	65.3
Stage II						
Shanghai-II (IIa)	Chinese	1,613	1,800	53.2/53.4	50.2/55.1	62.5
SeBCS-I (IIb)	Korean	2,359	2,052	48.1/51.7	37.9/52.0	61.9
Stage III						
Shanghai-III	Chinese	2,601	2,386	53.8/55.1[d]	50.3/52.6	64.9
Taiwan	Chinese	1,066	1,065	51.5/47.5[d]	52.3/39.9	66.1
Nagoya	Japanese	644	644	51.4/51.1	48.5/48.5	72.8
Nagano	Japanese	401	401	53.8/54.0	54.9/65.3	74.6
Stage IV						
Nanjing	Chinese	1,786	1,837	50.6/50.2	51.3/47.6	55.7
Tianjin	Chinese	1,297	1,585	51.9/51.9	51.9/55.5	44.2
Guangzhou	Chinese	838	865	49.0/49.2	41.8/51.9	71.6
NCC	Korean	505	504	49.0/49.1	49.5/45.3	65.0
SeBCS-II	Korean	777	1,104	47.5/47.7	36.3/37.3	63.0
KOHBRA/KoGES	Korean	1,397	3,209	40.5/50.3[d]	23.3/	62.8
MEC	Japanese	889	830	66.5/66.5		85.3
Total		19,091	20,606			

[a]See the methods section for the full names of participating studies.
[b]Mean value for cases/controls.
[c]Percentage for cases/controls.
[d]Significant at $\alpha=0.01$ level.
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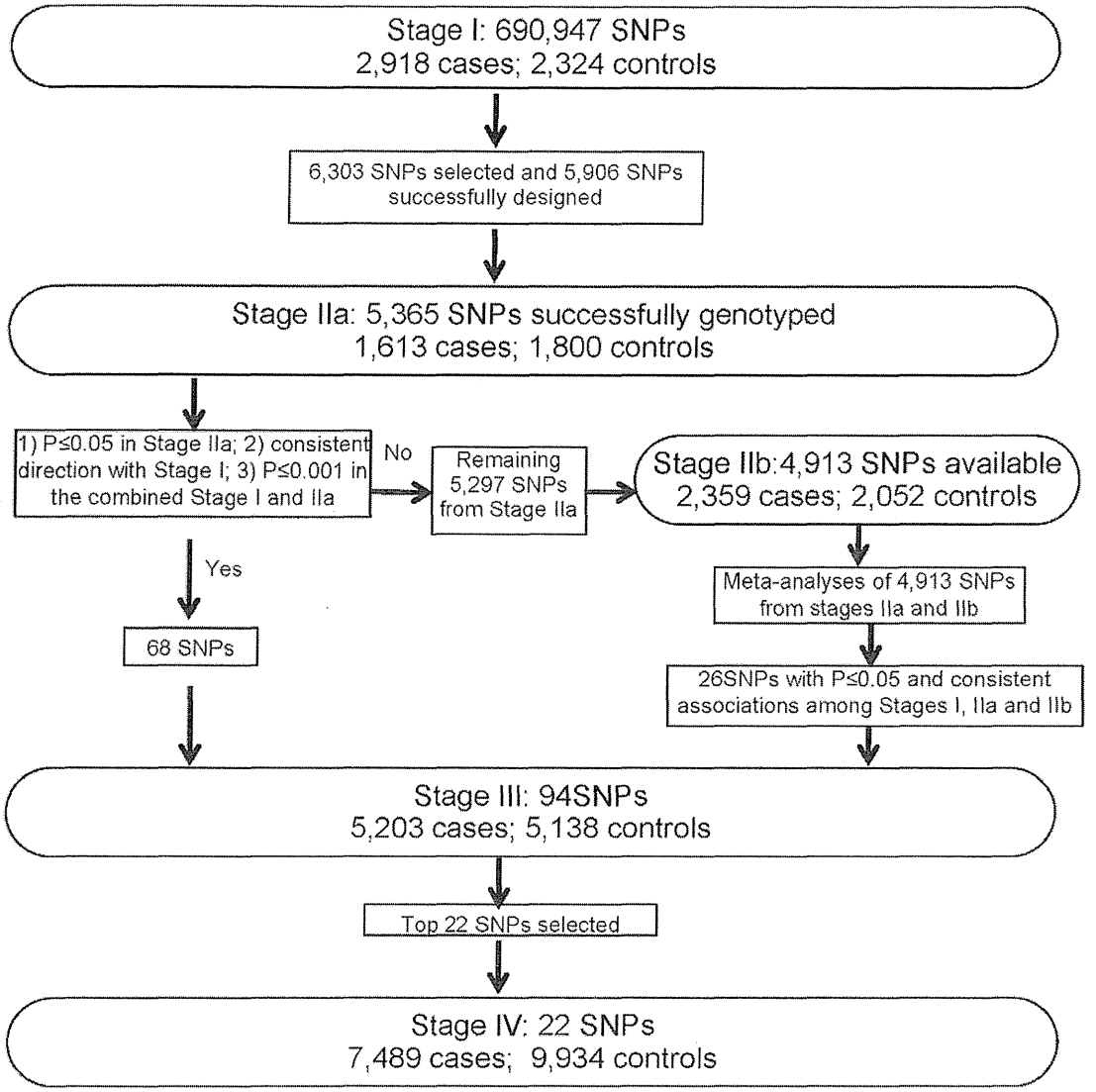

Figure 1. Overview of the study design.
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contribute to the development of cancer [20]. Furthermore, the TAB2 protein is required for DNA damage-induced TAK1 activation, suggesting that TAB2 may play a role in DNA damage repair [21]. Other genes in the region identified in the study included *SUMO4*, *LATS1*, *PPIL4*, and *UST*. However, given the proximity of the *TAB2* gene with rs9485372 and the important role of this gene in breast carcinogenesis, it is possible that the association between rs9485372 and breast cancer risk may be mediated through the *TAB2* gene. It is also possible that the association may be mediated through regulating the *ESR1* gene, located approximately 2.5 Mb from rs9485372. This possibility was highlighted by a recent study showing that several open reading frames in the 6q25.1 regions co-expressed with *ESR1* [22]. Further research is warranted to clarify the mechanism of the association identified in the study.

SNP rs9383951 is located in intron 5 of the *ESR1* gene, an important gene that has been documented to play a key role in breast cancer development and progression. Previous candidate gene studies have extensively evaluated two SNPs, rs2234693 (PvuII) and rs9340799 (XbaI), in the *ESR1* gene in relation to breast cancer risk; the results, however, have been inconsistent [2]. Neither rs2234693 nor rs9340799 are in LD ($r^2<0.01$) with the SNPs discovered in the present study. To follow-up the lead from our previous study reporting a susceptibility locus at 6q25.1 for breast cancer [8], two recent studies conducted among women of European descent identified rs3757318 and rs9397435 in relation to breast cancer risk [11,23]. These two SNPs are in strong LD ($r^2>0.6$ in Asians) with the SNP (rs2046210) we previously reported at 6q25.1 in East Asians but not in other populations. Again, these two SNPs are not in LD ($r^2<0.01$ in Asian, European and African populations) with rs9383951 and rs9485372 identified in this study. Although the association with rs9383951 did not reach the conventional genome-wide significance, the fact that this SNP is located in the *ESR1* gene strongly suggests a true association of this SNP with breast cancer risk.

Table 2. Summary of results for the three SNPs showing a statistically or marginally significant association in all four stages with breast cancer risk, the Asia Breast Cancer Consortium.

SNP[a]	Position[b]	Study	No. of Cases/Controls	EAF (%)[c]	Per allele OR (95%CI) d	P value[d]
rs9485372 (A/G)	149650567 (6q25.1)					
		Stage I	2,770/2,175	43.5	0.88(0.81–0.95)	1.4×10^{-3}
		Stage II	3,930/3,818	47.1	0.86(0.81–0.92)	6.3×10^{-6}
		Stage III	4,081/4,074	43.2	0.94(0.88–1.00)	0.05
		Stage IV	5,186/7,440	46.2	0.90(0.85–0.94)	4.2×10^{-5}
		All stages	15,967/17,507	45.4	0.90(0.87–0.92)	3.8×10^{-12}
rs9383951 (C/G)	152337306 (6q25.1)					
		Stage I	2,916/2,319	11.4	0.82(0.73–0.93)	2.4×10^{-3}
		Stage II	3,948/3,836	10.1	0.90(0.81–1.00)	0.06
		Stage III	4,581/4,433	9.7	0.91(0.82–1.00)	0.06
		Stage IV	6,117/8,296	9.6	0.88(0.81–0.96)	3.3×10^{-3}
		All stages	17,562/18,884	10.0	0.88(0.84–0.93)	1.9×10^{-6}
rs7107217 (C/A)	128978900 (11q24.3)					
		Stage I	2,916/2,319	31.4	1.13(1.04–1.23)	3.6×10^{-3}
		Stage II	3,929/3,839	34.8	1.11(1.04–1.18)	2.1×10^{-3}
		Stage III	4,606/4,424	35.2	1.07(1.00–1.14)	0.04
		Stage IV	7,348/9,831	37.4	1.05(1.01–1.10)	0.02
		All stages	18,799/20,413	35.8	1.08(1.05–1.11)	4.6×10^{-7}

[a]Effect/reference alleles based on forward strand.
[b]From NCBI genome build 36.
[c]Effect allele frequency in controls.
[d]Adjusted for age and study sites.
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SNP rs7107217 also showed a consistent association in all four stages, although the pooled *P*-value did not reach the conventional genome-wide significance level. This SNP is located at 11q24.3, 152 Kb downstream of the *BARX2* gene and 212 Kb upstream of the *TMEM45B* gene (Figure S3). *BARX2* is a homeobox gene for which the mouse ortholog has been shown to influence cellular processes that control cell adhesion and cytoskeleton remodeling. It has been shown, BARX2 and estrogen receptor-alpha (ESR1) coordinately regulate the production of alternatively spliced ESR1 isoforms and control breast cancer cell growth and invasion [24]. BARX2 also acts in a tumor suppressor and loss of heterozygosity of this gene, lead to poorer survival in patients with ovarian cancer [25].

It could be ideal to increase the sample size in the discovery stage and simplify the replication stages of the study. However, like many other consortium projects, financial constraints and some logistical issues prevented us for achieving the maximum statistical power. Nevertheless, with approximately 40,000 cases and controls, our study represents the largest breast cancer genetic association study in East Asian women. This consortium will continue to provide valuable resources to identify additional novel susceptibility loci for breast cancer.

In summary, in this large GWAS conducted in East Asia women, we provided convincing evidence for an association with a novel independent susceptibility locus located at 6q25.1, near the *TAB2* gene. Our study also suggests that genetic variants in the *ESR1* gene and chromosome 11q24.3 may be related to breast cancer risk. Given that multiple independent breast cancer susceptibility loci have identified in our studies and studies conducted by others in 6q25.1 that harbors the *ESR1* gene, it is possible that 6q25.1 may represent an important region for breast cancer susceptibility.

Methods

Study populations

Included in this consortium project were 19,091 cases and 20,606 controls from 14 studies (Table 1). Detailed descriptions of these participating studies and demographic characteristics of study participants are provided in Text S1. Briefly, the consortium included 23,981 Chinese women, 11,907 Korean women, 3,809 Japanese women. The Chinese women were from 8 studies: Shanghai [n = 13,642, Shanghai Breast Cancer Study, Shanghai Breast Cancer Survival Study (SBCSS), Shanghai Endometrial Cancer Study (SECS), Shanghai Women Health Study (SWHS)] [8,26], Nanjing (n = 3,623) [27], Tianjin (n = 2,882) [28], Taiwan (n = 2,131) [29], and Guangzhou (n = 1,703). The Korean women were from four studies [Seoul Breast Cancer Study (SeBCS) (n = 6,292) [30], Korea NCC (n = 1,009), KoGES (n = 3,209) [31], and KOHBRA (n = 1,397) [32]]. The Japanese women were from three studies conducted in Hawaii and Los Angeles [n = 1,719; Multiethnic Cohort Study (MEC) [33]], Nagoya (n = 1,288) [34], and Nagano (n = 802) [35] (Table 1). Approval was granted from relevant institutional review boards in all study sites; all included subjects gave informed consent.

Genotyping methods

The Genotyping protocol for Stage I has been described previously [8]. Briefly, the initial 300 subjects were genotyped using the Affymetrix GeneChip Mapping 500K Array Set. The remaining 4,985 subjects were genotyped using the Affymetrix Genome-Wide Human SNP Array 6.0. We included one negative control and at least three positive quality control (QC) samples from the Coriell Cell Repositories (http://ccr.coriell.org/) in each

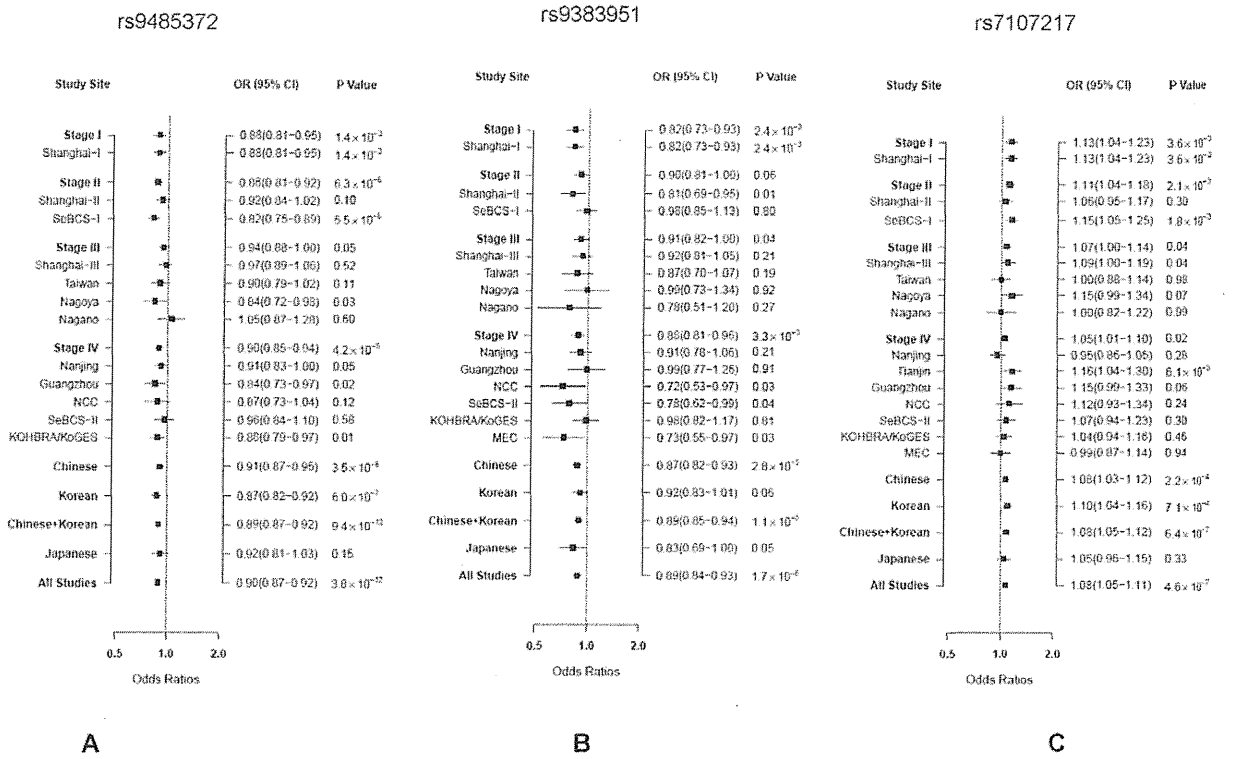

Figure 2. ORs per risk allele and 95% CIs for breast cancer associated with three SNPs by study site and ethnicity. A: rs9485372, B: rs9383951; and C: rs7107217.
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Table 3. Association of SNPs with breast cancer risk by ethnic groups, the Asia Breast Cancer Consortium.

SNP	Study	No. of Cases/Controls	EAF (%)[a]	OR (95% CI)[b]		P value[b]
				Heterozygote	Homozygote	
rs9485372						
	Chinese	9,922/9,644	43.2	0.90(0.84–0.96)	0.83(0.76–0.90)	3.5×10^{-6}
	Korean	5,006/6,825	48.2	0.87(0.79–0.95)	0.76(0.68–0.85)	6.0×10^{-7}
	Chinese+Korean	14,928/16,469	45.2	0.89(0.85–0.94)	0.80(0.75–0.85)	9.4×10^{-12}
	Japanese	1,039/1,038	47.5	0.93(0.76–1.13)	0.84(0.66–1.07)	0.15
	All studies	15,967/17,507	45.4	0.89(0.85–0.94)	0.80(0.75–0.86)	3.8×10^{-12}
rs9383951						
	Chinese	10,625/10,180	10.7	0.86(0.80–0.92)	0.87(0.67–1.13)	3.4×10^{-5}
	Korean	5,011/6,833	9.7	0.92(0.83–1.02)	0.79(0.52–1.19)	0.06
	Chinese+Korean	15,636/17,013	10.3	0.88(0.83–0.93)	0.86(0.69–1.07)	1.3×10^{-5}
	Japanese	1,926/1,871	6.8	0.86(0.71–1.05)	0.40(0.14–1.13)	0.05
	All studies	17,562/18,884	10.0	0.88(0.83–0.93)	0.83(0.67–1.03)	1.9×10^{-6}
rs7107217						
	Chinese	11,887/11,719	32.3	1.09(1.03–1.15)	1.14(1.05–1.25)	2.2×10^{-4}
	Korean	4,987/6,824	38.7	1.13(1.04–1.23)	1.19(1.06–1.34)	7.1×10^{-4}
	Chinese+Korean	16,874/18,543	34.6	1.10(1.05–1.15)	1.16(1.08–1.24)	6.4×10^{-7}
	Japanese	1,925/1,870	47.3	1.09(0.94–1.27)	1.09(0.91–1.31)	0.33
	All studies	18,799/20,413	35.8	1.10(1.05–1.15)	1.15(1.08–1.22)	4.6×10^{-7}

[a]Effect allele frequency in controls.
[b]Adjusted for age and study sites.
doi:10.1371/journal.pgen.1002532.t003

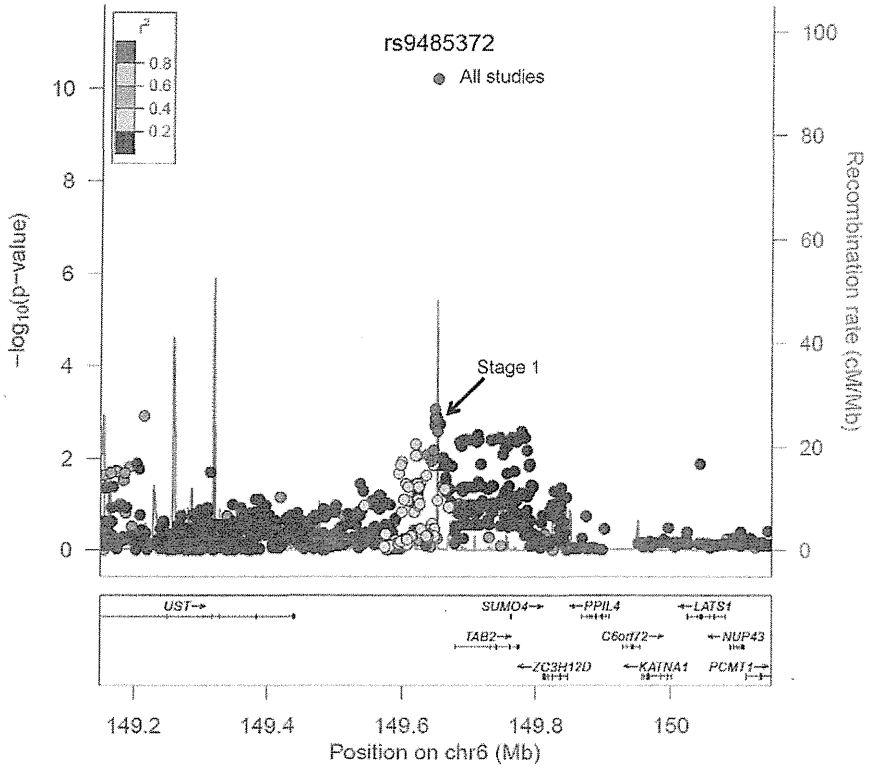

Figure 3. A regional plot of the $-\log_{10}$P-values for SNPs at 6q25.1. The LD is estimated using data from HapMap Asian population. Also shown are the SNP Build 36 coordinates in kilobases (Kb), recombination rates in centimorgans (cM) per megabase (Mb) and genes in the region (below) based on the March 2006 UCSC genome browser assembly.
doi:10.1371/journal.pgen.1002532.g003

of the 96-well plates for Affymetrix SNP Array 6.0 genotyping. A total of 273 positive QC samples were successfully genotyped, and the average concordance rate was 99.9% with a median value of 100%. The sex of all study samples was confirmed to be female. Genetically identical, unexpected duplicated samples were excluded, as were close relatives with a pair-wise proportion of identify-by-descent (IBD) estimate greater than 0.25. All samples with a call rate<95% were excluded. The SNPs were excluded if: (i) MAF<1%, (ii) call rate<95%, or (iii) genotyping concordance rate<95% in quality control samples. The final dataset included 2,918 cases and 2,324 controls for 690,947 markers. There are 21,223 SNPs that were on Affymetrix 500K Array Set but not on the Affymetrix SNP Array 6.0. These SNPs were excluded. SNPs on the Affymetrix 6.0 array but not on the Affymetrix 500k array were treated as missing data for those samples genotyped on using the Affymetrix 500k array. Similar results were obtained after excluding women genotyped by Affymetrix 500K Array Set from the analyses.

Genotyping for Stage IIa was completed using the Illumina iSelect platform. To compare the consistency between the Affymetrix and Illumina iSelect platforms, we also included 43 samples from Stage I that were genotyped by Affymetrix SNP 6.0. Similar to the QC procedures used in Stage I, the following criteria were used to exclude samples: (i) call rate<95%; or (ii) unexpected duplicated samples based on IBD estimate. SNPs were excluded if: (i) call rate<95%, or (ii) genotyping concordance rate<95% in quality control samples when compared with Affymetrix 6.0 data. After QC, the mean concordance rate was 99.85% between Illumina iSelect and Affymetrix 6.0 genotyping.

Data for the SNPs analyzed in Stage IIb were extracted from the Korean GWAS genotyped using the Affymetrix Genome-Wide Human SNP Array 6.0 chip. A total of 30 QC samples were successfully genotyped, and the concordance rate was 99.83%. The sex of all samples was confirmed to be female. The SNPs were excluded if: (1) genotype call rate<95%, (2) MAF<1% in either the cases or controls, (3) deviation from HWE at P-value$<10^{-6}$, and (4) poor cluster plot in either the cases or controls.

Genotyping for Stage III and all samples from Koreans in Stage IV was completed using the iPLEX Sequenom MassArray platform in the Vanderbilt Molecular Epidemiology Laboratory. Included in each 96-well plate as QC samples were one negative control (water), two blinded duplicates, and two samples from the HapMap project. To compare the consistency between the Affymetrix and Sequenom platforms, we also genotyped 45 samples included in Stage I. The mean concordance rate was 99.67% for the blind duplicates, 98.88% for HapMap samples, and 99.52% between Sequenom and Affymetrix 6.0 genotyping. Data quality from the Hong Kong study was low and thus data from the study were excluded for the current analysis. Genotyping for two Chinese studies (Nanjing and Guangzhou) in Stage IV was completed using the iPLEX Sequenom MassArray platform at the Fudan University, Shanghai, China. Blind duplicate QC samples were included and the mean concordance rate was 98.70%. Genotyping for the Tianjin study in Stage IV was performed using TaqMan assays. Genotyping assay protocols were developed and validated at the Vanderbilt Molecular Epidemiology Laboratory, and TaqMan genotyping assay reagents were provided to investigators of the Tianjin study (Tianjin Cancer Institute and

Hospital). For the MEC study, data for the three SNPs presented in this study were extracted from the GWA scan data generated using Illumina 660W. For SNPs not included on the chip, imputed data using HapMap as reference were extracted. Genotype frequencies for SNP rs9485372 deviated from HWE in controls (P = 0.004), therefore, this SNP was excluded in data analyses. Not all SNPs for Stage IV were genotyped in all studies included in Stage IV due to genotyping failure or the use of different genotyping platforms (Table S8).

SNP selection for replication

SNP selection for Stage II replication: Promising SNPs were selected for replication in Stage II based on the following criteria: 1) minor allele frequency (MAF)≥5%; 2) $P<0.02$ in Stage I; 3) Hardy-Weinberg equilibrium (HWE) test $P>1.0\times10^{-6}$ in controls; 4) not in strong linkage disequilibrium (LD) ($r^2<0.5$) with any of the previously confirmed breast cancer genetic risk variants or SNPs evaluated in our previous studies [8,12]; and 5) high genotyping quality as indicated by very clear genotyping clusters checked manually. When multiple SNPs are in LD with $r^2\geq0.5$, one SNP with the lowest *P*-value was selected. In total, 6,303 SNPs were selected for replication. A total of 5,906 SNPs (93.7%) were successfully designed by Illumina and included in the iSelect array. After stringent QC procedures, data from 5,365 SNPs were considered high quality for association analyses in Stage IIa, which include 1,613 breast cancer patients and 1,800 controls recruited from Shanghai studies.

SNP selection for Stage III replication: Among the 5,365 SNPs successfully genotyped in Stage IIa, 68 SNPs were selected for Stage III replication in an independent set of 5,203 cases and 5,138 controls recruited from Shanghai and several other East Asian populations (Table 1 and Text S1). The selection criteria are: 1) an association with breast cancer risk in Stage IIa with P≤0.05; 2) the direction of the association consistent in both stages; and 3) P≤0.001 in the merged data of Stage I and IIa. During the course of Stage III genotyping, genome-wide association scan data from 2,359 cases and 2,052 controls were obtained from the Seoul Breast Cancer GWAS (Stage IIb). Therefore, we performed a meta-analysis of Stage IIa and IIb data. Of the 5,297 SNPs which were not selected initially for Stage III replication based on Stage IIa data alone, data were available for 4,913 SNPs in Stage IIb. Meta-analyses of these 4,913 SNPs from Stage IIa and IIb yielded 26 additional SNPs that showed an association at $P\leq0.05$ and in the same direction among stages I, IIa, and IIb. These 26 SNPs were then added to the list of SNPs to be genotyped in Stage III.

SNP selection for Stage IV replication: Based on the results of the first three stages, 22 top SNPs were selected for Stage IV evaluation and genotyped in up to 17,423 additional subjects (7,489 cases and 9,934 controls) (Table 1 and Text S1).

Statistical analyses

Case-control differences in selected demographic characteristics and major risk factors were evaluated using t-tests (for continuous variables) and Chi-square tests (for categorical variables). Associations between SNPs and breast cancer risk were assessed using odds ratios (ORs) and 95% confidence intervals (CIs) derived from logistic regression models. ORs were estimated for heterozygote and homozygote for the variant allele compared with homozygotes for the common allele. ORs were also estimated for the variant allele based on a log-additive model and adjusted for age, and study site, when appropriate. Stratified analyses by ethnicity, menopausal status, and estrogen receptor (ER) status were carried out. PLINK version 1.06 was used to analyze genome-wide data obtained in Stage I and the replication data in Stage IIa. Results from Stage IIb were also obtained from PLINK version 1.06. Meta-analyses of Stage IIa and Stage IIb were performed using a weighted z-statistics method, where weights were proportional to the square root of the number of individuals in each sample and standardized such that the weights added up to one. The z-statistic

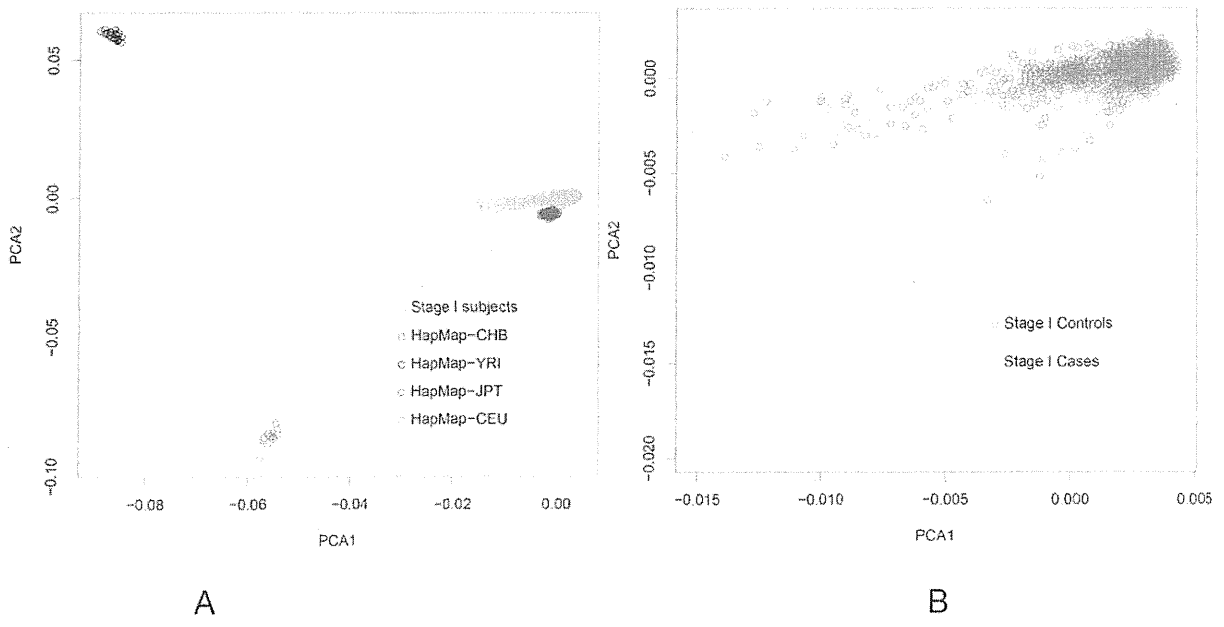

Figure 4. Principal Component Analysis (PCA) based on the first two eigenvectors obtained by PCA. A: all individuals from Stage I and HapMap; B: breast cancer cases and controls from Stage I.
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summarizes the magnitude and direction of the effect relative to the reference allele. An overall z-statistic and p value were then calculated from the weighted average of the individual statistics. Calculations were implemented in the METAL package (http://www.sph.umich.edu/csg/abecasis/Metal). Individual data were obtained from each study for Stage IV SNPs for a pooled analysis, which were conducted using SAS, version 9.2, with the use of two-tailed tests.

We first investigated the population structure by estimating inflation factor λ using all 690,947 SNPs SNPs that passed the QC. The inflation factor λ was estimated to be 1.042, suggesting that any population substructure, if present, should not have any appreciable effect on the results. Among the final 690,947 SNPs obtained in Stage I after QC, we generated a list of 196,471 SNPs with pairwise LD<0.2 by using plink (http://pngu.mgh.harvard.edu/~purcell/plink/). Then, principal components were estimated based on these 196,471 SNPs using EIGENSTRAT [36]. We then drew a plot for all Stage I and HapMap II subjects based on the first two principal components (Figure 4). All study participants in Stage I were clustered very closely with HapMap Asians. The first 5 or 10 principal components were adjusted in the logistic regression analyses for evaluating associations of SNPs and breast cancer risk.

To evaluate the combined effect of SNPs located in chromosome 6q25.1 on breast cancer risk, we created a genetic risk score (GRS) by summing the number (0–2) of risk alleles that each woman carried for each of the three SNPs, including rs9383951, rs9485372, rs2046210. The GRS was constructed among those who had complete data for all three SNPs. We also did imputation using MACH (http://www.sph.umich.edu/csg/abecasis/MACH/index.html) with HapMap II Asian data as reference. LD structure was estimated from the flanking 100 kb of these three SNPs and the *ESR1* gene using data from HapMap II Asians (Figure S1). All SNPs in the LD blocks including rs9485372, rs2046210 and rs9383951 and SNPs inside the *ESR1* gene were analyzed in relation to breast cancer risk with age, rs9485372, rs9383951 and rs2046210 adjusted.

Supporting Information

Figure S1 Estimates of pairwise LD (r^2) for common SNPs from HapMap II Asians for the SNPs located in 6q25.1. A: LD plot for the flanking 100 kb of SNP rs9485372. B: LD plot for the upstream 100 kb of SNP rs2046210 and the ESR1 gene.
(TIF)

Figure S2 Estimates of pairwise LD (r^2) from HapMap II Asian for the SNPs showing significant associations after adjusted for rs9485372, rs9383951 and rs2046210.
(TIF)

Figure S3 A regional plot of the $-\log_{10}$P-values for SNPs at 11q24.3. The LD is estimated using data from HapMap Asian population. Also shown are the SNP Build 36 coordinates in kilobases (Kb), recombination rates in centimorgans (cM) per megabase (Mb) and genes in the region (below) based on the March 2006 UCSC genome browser assembly.
(TIF)

Table S1 Association of SNPs with breast cancer risk by menopause and ER status.
(DOCX)

Table S2 Association results adjusted for the top principal components in Stage I.
(DOCX)

Table S3 LD between the 3 SNPs that are associated with breast cancer and are located in 6q25.1.
(DOCX)

Table S4 Conditional analyses for SNPs located on 6q25.1.
(DOCX)

Table S5 Association results of SNP-SNP interaction.
(DOCX)

Table S6 Associations of breast cancer risk with the genetic risk score for the three SNPs located in chromosome 6q25.1, the Asia Breast Cancer Consortium.
(DOCX)

Table S7 SNPs in 6q25.1 showed association after adjusted for rs9485372, rs9383951 and rs2046210.
(DOCX)

Table S8 Sample size for the SNPs included in Stage IV.
(DOCX)

Text S1 Supplementary Methods.
(DOCX)

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Author Contributions

Conceived and designed the overall study: W Zheng. Performed genotyping experiments: J Shi, H Zheng. Wrote the manuscript: J Long, W Zheng, Q Cai, X-O Shu. Significantly contributed to writing the manuscript: C Li, W Wen, RJ Delahanty. Coordinated genotyping assays: Q Cai, J Long. Managed genotyping data: J Long, B Zhang. Performed statistical analyses: J Long, C Li, W Wen. Directed lab operations: Q Cai. Directed the GWAS in Korea: D-H Kang. Assisted the GWAS in Korea: H Sung, J-Y Choi. Contributed to data and biological collection of the original studies: H Shen, J-Y Choi, W Lu, Y-T Gao, H Shen, SK Park, K Chen, C-Y Shen, Z Ren, CA Haiman, K Matsuo, MK Kim, US Khoo, M Iwasaki, Y Zheng, Y-B Xiang, K Gu, N Rothman, W Wang, Z Hu, Y Liu, K-Y Yoo, D-Y Noh, B-G Han, MH Lee, H Zheng, L Zhang, P-E Wu, Y-L Shieh, SY Chan, S Wang, X Xie, S-W Kim, BE Henderson, L Le Marchand, H Ito, Y Kasuga, S-H Ahn, HS Kang, KYK Chan, H Iwata, S Tsugane, D-H Kang, X-O Shu, W Zheng.

References

1. Nathanson KL, Wooster R, Weber BL (2001) Breast cancer genetics: what we know and what we need. Nat Med 7: 552–556.
2. Zhang B, Beeghly-Fadiel A, Long J, Zheng W (2011) Genetic variants associated with breast-cancer risk: comprehensive research synopsis, meta-analysis, and epidemiological evidence. Lancet Oncol 12: 477–488.
3. Easton DF, Pooley KA, Dunning AM, Pharoah PD, Thompson D, et al. (2007) Genome-wide association study identifies novel breast cancer susceptibility loci. Nature 447: 1087–1093.
4. Hunter DJ, Kraft P, Jacobs KB, Cox DG, Yeager M, et al. (2007) A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer. Nat Genet 39: 870–874.
5. Stacey SN, Manolescu A, Sulem P, Rafnar T, Gudmundsson J, et al. (2007) Common variants on chromosomes 2q35 and 16q12 confer susceptibility to estrogen receptor-positive breast cancer. Nat Genet 39: 865–869.

6. Gold B, Kirchhoff T, Stefanov S, Lautenberger J, Viale A, et al. (2008) Genome-wide association study provides evidence for a breast cancer risk locus at 6q22.33. Proc Natl Acad Sci U S A 105: 4340–4345.
7. Stacey SN, Manolescu A, Sulem P, Thorlacius S, Gudjonsson SA, et al. (2008) Common variants on chromosome 5p12 confer susceptibility to estrogen receptor-positive breast cancer. Nat Genet 40: 703–706.
8. Zheng W, Long J, Gao YT, Li C, Zheng Y, et al. (2009) Genome-wide association study identifies a new breast cancer susceptibility locus at 6q25.1. Nat Genet 41: 324–328.
9. Thomas G, Jacobs KB, Kraft P, Yeager M, Wacholder S, et al. (2009) A multistage genome-wide association study in breast cancer identifies two new risk alleles at 1p11.2 and 14q24.1 (RAD51L1). Nat Genet 41: 579–584.
10. Ahmed S, Thomas G, Ghoussaini M, Healey CS, Humphreys MK, et al. (2009) Newly discovered breast cancer susceptibility loci on 3p24 and 17q23.2. Nat Genet 41: 585–590.
11. Turnbull C, Ahmed S, Morrison J, Pernet D, Renwick A, et al. (2010) Genome-wide association study identifies five new breast cancer susceptibility loci. Nat Genet 42: 504–507.
12. Long J, Cai Q, Shu XO, Qu S, Li C, et al. (2010) Identification of a functional genetic variant at 16q12.1 for breast cancer risk: results from the Asia Breast Cancer Consortium. PLoS Genet 6: e1001002. doi:10.1371/journal.pgen.1001002.
13. Antoniou AC, Wang X, Fredericksen ZS, McGuffog L, Tarrell R, et al. (2010) A locus on 19p13 modifies risk of breast cancer in BRCA1 mutation carriers and is associated with hormone receptor-negative breast cancer in the general population. Nat Genet % 19.
14. Fletcher O, Johnson N, Orr N, Hosking FJ, Gibson LJ, et al. (2011) Novel breast cancer susceptibility locus at 9q31.2: results of a genome-wide association study. J Natl Cancer Inst 103: 425–435.
15. Fletcher O, Houlston RS (2010) Architecture of inherited susceptibility to common cancer. Nat Rev Cancer 10: 353–361.
16. Zheng W, Cai Q, Signorello LB, Long J, Hargreaves MK, et al. (2009) Evaluation of 11 breast cancer susceptibility loci in African-American women. Cancer Epidemiol Biomarkers Prev 18: 2761–2764.
17. Zheng W, Wen W, Gao YT, Shyr Y, Zheng Y, et al. (2010) Genetic and clinical predictors for breast cancer risk assessment and stratification among Chinese women. J Natl Cancer Inst 102: 972–981.
18. Long J, Shu XO, Cai Q, Gao YT, Zheng Y, et al. (2010) Evaluation of breast cancer susceptibility loci in Chinese women. Cancer Epidemiol Biomarkers Prev 19: 2357–2365.
19. Benson JR (2004) Role of transforming growth factor beta in breast carcinogenesis. Lancet Oncol 5: 229–239.
20. Davis RJ (2000) Signal transduction by the JNK group of MAP kinases. Cell 103: 239–252.
21. Hinz M, Stilmann M, Arslan SC, Khanna KK, Dittmar G, et al. (2010) A cytoplasmic ATM-TRAF6-cIAP1 module links nuclear DNA damage signaling to ubiquitin-mediated NF-kappaB activation. Mol Cell 40: 63–74.
22. Dunbier AK, Anderson H, Ghazoui Z, Lopez-Knowles E, Pancholi S, et al. (2011) ESR1 Is Co-Expressed with Closely Adjacent Uncharacterised Genes Spanning a Breast Cancer Susceptibility Locus at 6q25.1. PLoS Genet 7: e1001382. doi:10.1371/journal.pgen.1001382.
23. Stacey SN, Sulem P, Zanon C, Gudjonsson SA, Thorleifsson G, et al. (2010) Ancestry-shift refinement mapping of the C6orf97-ESR1 breast cancer susceptibility locus. PLoS Genet 6: e1001029. doi:10.1371/journal.pgen.1001029.
24. Stevens TA, Meech R (2006) BARX2 and estrogen receptor-alpha (ESR1) coordinately regulate the production of alternatively spliced ESR1 isoforms and control breast cancer cell growth and invasion. Oncogene 25: 5426–5435.
25. Sellar GC, Li L, Watt KP, Nelkin BD, Rabiasz GJ, et al. (2001) BARX2 induces cadherin 6 expression and is a functional suppressor of ovarian cancer progression. Cancer Res 61: 6977–6981.
26. Gao YT, Shu XO, Dai Q, Potter JD, Brinton LA, et al. (2000) Association of menstrual and reproductive factors with breast cancer risk: results from the Shanghai Breast Cancer Study. Int J Cancer 87: 295–300.
27. Liang J, Chen P, Hu Z, Zhou X, Chen L, et al. (2008) Genetic variants in fibroblast growth factor receptor 2 (FGFR2) contribute to susceptibility of breast cancer in Chinese women. Carcinogenesis 29: 2341–2346.
28. Zhang L, Gu L, Qian B, Hao X, Zhang W, et al. (2009) Association of genetic polymorphisms of ER-alpha and the estradiol-synthesizing enzyme genes CYP17 and CYP19 with breast cancer risk in Chinese women. Breast Cancer Res Treat 114: 327–338.
29. Ding SL, Yu JC, Chen ST, Hsu GC, Kuo SJ, et al. (2009) Genetic variants of BLM interact with RAD51 to increase breast cancer susceptibility. Carcinogenesis 30: 43–49.
30. Choi JY, Lee KM, Park SK, Noh DY, Ahn SH, et al. (2005) Association of paternal age at birth and the risk of breast cancer in offspring: a case control study. BMC Cancer 5: 143.
31. Cho YS, Go MJ, Kim YJ, Heo JY, Oh JH, et al. (2009) A large-scale genome-wide association study of Asian populations uncovers genetic factors influencing eight quantitative traits. Nat Genet 41: 527–534.
32. Han SA, Park SK, Hyun AS, Hyuk LM, Noh DY, et al. (2011) The Korean Hereditary Breast Cancer (KOHBRA) Study: Protocols and Interim Report. Clin Oncol (R Coll Radiol).
33. Kolonel LN, Henderson BE, Hankin JH, Nomura AM, Wilkens LR, et al. (2000) A multiethnic cohort in Hawaii and Los Angeles: baseline characteristics. Am J Epidemiol 151: 346–357.
34. Hamajima N, Matsuo K, Saito T, Hirose K, Inoue M, et al. (2001) Gene-environment Interactions and Polymorphism Studies of Cancer Risk in the Hospital-based Epidemiologic Research Program at Aichi Cancer Center II (HERPACC-II). Asian Pac J Cancer Prev 2: 99–107.
35. Itoh H, Iwasaki M, Hanaoka T, Kasuga Y, Yokoyama S, et al. (2009) Serum organochlorines and breast cancer risk in Japanese women: a case-control study. Cancer Causes Control 20: 567–580.
36. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, et al. (2006) Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet 38: 904–909.

Low-dose carcinogenicity of 2-amino-3-methylimidazo[4,5-*f*]quinoline in rats: Evidence for the existence of no-effect levels and a mechanism involving p21$^{Cip/WAF1}$

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The carcinogenicity of the low amounts of genotoxic carcinogens present in food is of pressing concern. The purpose of the present study was to determine the carcinogenicity of low doses of the dietary genotoxic carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and to investigate mechanisms by which IQ exerts its carcinogenic effects. A total of 1595 male F344 rats were divided into seven groups and administered with IQ at doses of 0, 0.001, 0.01, 0.1, 1, 10 and 100 p.p.m. in the diet for 16 weeks. We found that IQ doses of 1 p.p.m. and below did not induce preneoplastic lesions in either the liver or the colon, while IQ doses of 10 and 100 p.p.m. induced preneoplastic lesions in both of these organs. These results demonstrate the presence of no-effect levels of IQ for both liver and colon carcinogenicity in rats. The finding that p21$^{Cip/WAF1}$ was significantly induced in the liver at doses well below those required for IQ mediated carcinogenic effects suggests that induction of p21$^{Cip/WAF1}$ is one of the mechanisms responsible for the observed no-effect of low doses of IQ. Furthermore, IQ administration caused significant induction of CYP1A2 at doses of 0.01–10 p.p.m., but administration of 100 p.p.m. IQ induced CYP1A1 rather than CYP1A2. This result indicates the importance of dosage when interpreting data on the carcinogenicity and metabolic activation of IQ. Overall, our results suggest the existence of no-effect levels for the carcinogenicity of this genotoxic compound. (*Cancer Sci* 2011; 102: 88–94)

Exposure to environmental carcinogens is one of the most significant causes of human cancers. Determination of the dose-response relationship between carcinogen exposure and induction of cancer is one of the most important areas of chemical risk assessment. Of particularly high priority is the cancer risk assessment of dietary carcinogens.

Heterocyclic amines (HCA) are well known dietary genotoxic carcinogens derived from cooked protein-rich foods such as meat and fish,[1–3] and the carcinogenicities of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) have been widely investigated in various animal models. MeIQx induces cancers of the liver, zymbal gland, skin and clitoral gland in rats,[4] and caners of the liver and lung, and lymphoma and leukemia in mice.[5] PhIP induces colon cancers and mammary gland cancers in rats,[6] and lymphomas in mice.[7] IQ induces cancers of the liver, colon, mammary and zymbal glands in rats, caners of the liver, lung and forestomach in mice, and cancer of the liver in non-human primates.[8–10] MeIQx and PhIP are classified as category 2B compounds (possibly carcinogenic to humans) and IQ is classified as a category 2A compound (probably carcinogenic to humans) by the International Agency for Research on Cancer.[11] Therefore, although the concentrations of HCA in food are low, they constitute a potential hazard, and there is concern regarding the carcinogenic effects of low doses of these HCA.

Based on the view that even minute doses of a genotoxic carcinogen has the potential to produce irreversible deleterious genetic changes in the DNA of a target organ cell and the argument that if sufficient numbers of test animals are used the carcinogenic effect of a minute dose can be demonstrated, it is generally assumed that genotoxic carcinogens exert a non-threshold carcinogenic effect. However, the carcinogenicities of most genotoxic carcinogens are determined by experimental animal carcinogenicity studies using doses that are generally orders of magnitude higher than actual human exposure levels and the dose-response curves obtained are then extrapolated to zero using a non-threshold mathematical model. This approach, however, is being challenged as advancements in the understanding of the molecular mechanisms of carcinogenesis are being made and experimental evidence showing that genotoxic carcinogens do not exert mutagenic and carcinogenic effects at low doses accumulates.[12–19]

Previously, we demonstrated the existence of no-effect levels of MeIQx for both hepatocarcinogenicity and *in vivo* mutagenicity in various carcinogenesis models in different rat strains.[17,20–22] It has also been shown that low doses of PhIP do not exert either initiation or promotion activities in colon carcinogenesis in the rat.[23,24] However, little is known about the carcinogenic potential of low doses of IQ.

In addition, little is known about the mechanisms underlying the carcinogenicities of lower doses of HCA, but incorporation of mechanistic information is critical for quantitative cancer risk assessment. The purpose of the present study is to determine the relationship between administration of low doses of IQ and induction of preneoplastic lesions in the liver and colon in rats, and to investigate carcinogenic mechanisms of action of various doses of IQ by evaluating DNA-adduct formation, oxidative DNA damage and expression levels of genes involved in metabolic activation of IQ, cell proliferation and DNA damage repair in the liver.

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Materials and Methods

Chemical and diets. IQ was purchased from Nard Institute Ltd (Osaka, Japan) with a purity of 99.9%. Basal diets (powdered MF; Oriental Yeast Co., Tokyo, Japan) and the diets containing IQ were prepared once a month by Oriental Yeast Co.

Animals. A total of 1595 male F344 rats were supplied by Charles River Japan, Inc. (Hino, Shiga, Japan) and were used at 21 days of age. Animals were housed in polycarbonate cages (five per cage) in experimental animal rooms with a targeted temperature of 22 ± 3°C, relative humidity of 55 ± 5% and a 12-h light/dark cycle. Diet and tap water were available *ad libitum* throughout the study.

Experimental design. The animal experiment protocols were approved by the Institutional Animal Care and Use Committee of Osaka City University Medical School. Rats were randomized into seven groups, 245 rats in each of groups 1–6 and 125 rats in group 7. Since the levels of IQ in cooked foods are lower than those of MeIQx and PhIP,[11] IQ dosage and treatment duration in this study were the same as the previous low dose carcinogenicity studies with MeIQx and PhIP.[18,24] Animals were fed diets containing IQ as follows: 0 (group 1, control), 0.001 (group 2), 0.01 (group 3), 0.1 (group 4), 1 (group 5), 10 (group 6) and 100 p.p.m. (group 7) for 16 weeks. Fresh diet was supplied to the animals twice weekly. Bodyweights, food consumption and water intake were measured weekly.

Five rats in each group were killed at week 4 under ether anesthesia. At death, livers were snap frozen in liquid nitrogen and stored at −80°C for examination of IQ-DNA adducts and 8-hydroxy-2′-deoxyguanosine (8-OHdG) formation in the DNA. The remaining rats were killed at the end of week 16 under ether anesthesia for examination of the development of glutathione *S*-transferase placental form (GST-P) positive foci, which is a well-established preneoplastic lesion in the rat liver,[25,26] and aberrant crypt foci (ACF), which is a surrogate marker for preneoplastic lesions in the rat colon.[24,27,28] At death, livers were excised, weighed and then three slices each from the left lateral, medial and right lateral lobes were cut and placed in 10% phosphate-buffered formalin. The remaining liver tissues were snap frozen in liquid nitrogen and stored at −80°C for mRNA expression analysis. Following fixation, liver tissues were embedded in paraffin and processed for histopathological examination.

Examination of GST-P positive foci in the liver. Anti-rat GST-P polyclonal antibody (Medical and Biological Laboratories Co., Ltd, Nagoya, Japan) at a dilution of 1:1000 was used for immunohistochemical staining of GST-P. The GST-P-positive hepatocellular foci composed of two or more cells were counted under a light microscope.[17,18,20,22] Total areas of livers were measured using a color image processor IPAP (Sumica Technos, Osaka, Japan) and the number of GST-P-positive foci per square centimeter of liver tissue was calculated.

IQ-DNA adduct and 8-OHdG formation in livers. IQ-DNA adducts were measured by the ^{32}P-postlabeling method as described previously.[29,30] Levels of 8-OHdG formation in liver DNA were determined by high-performance liquid chromatography with electrochemical detection as previously described.[31]

TaqMan real-time quantitative PCR. The mRNA expression levels of genes involved in IQ metabolism (CYP1A1, CYP1A2 and CYP1B1), DNA damage repair (8-oxoguanine DNA glycosylase [Ogg1], growth arrest and DNA damage-inducible protein 45 [GADD45], AP endonuclease-1 [APE-1], MSH2 and MSH3) and cell cycle regulation (p53 and p21$^{Cip/WAF1}$ and proliferating cell nuclear antigen [PCNA]) were evaluated in the livers by TaqMan real-time quantitative PCR as described previously.[31] Sequence-specific primers and probes (Taqman Gene Expression Assay) were purchased from Applied Biosystems, Inc., Carlsbad, CA, USA. Beta-2-microglobulin (B2M) was used as an internal control.

Examination of ACF in colon. Formation of ACF was examined as described previously.[24] Although ACF consisting of four or more crypts are considered to be better predictors of colon tumor outcome in rats,[32] to ensure that all doses of IQ that have the potential to induce colon carcinogenesis were accounted for, doses of IQ that caused an increase of any size of ACF were considered to have the potential to induce colon carcinogenesis in the present study.[24]

Statistical analysis. All mean values are reported as mean ± SD. Statistical analyses were performed using the Statlight program (Yukms Co., Ltd, Tokyo, Japan). Homogeneity of variance was tested by the Bartlett test. Differences in mean values between the control and IQ-treated groups were evaluated by the 2-tailed Dunnett test when variance was homogeneous and the 2-tailed Steel test when variance was heterogeneous.[22,31] P values <0.05 were considered significant.

Results

General observation. All animals survived to the end of study without any apparent abnormal pathological features. The final average body and liver weights and IQ intake are summarized in Table 1. The final bodyweight of the 100 p.p.m. group was significantly lower than that of the 0 p.p.m. group. Absolute and relative liver weights were significantly decreased in the 0.1 and 1 p.p.m. groups and were significantly increased in the 100 p.p.m. group compared with the 0 p.p.m. group. There were no significant differences in either food or water consumption among groups (data not shown). The intake of IQ was proportional to the administered doses (Table 1). No tumors were found in any organs including the liver and colon in any of the groups.

Induction of GST-P-positive foci in the livers. No histopathological changes were observed in any of the IQ-treated groups.

Table 1. Body and organ weights, and IQ intake

Group	IQ (p.p.m.)	No. rats	Bodyweight (g)	Liver		Average IQ intake	
				Absolute weight (g)	Relative weight (%)	Daily intake (mg/kg b.w.)	Total (mg/kg b.w.)
1	0	240	331 ± 23	9.3 ± 1.7	2.8 ± 0.4	0	
2	0.001	240	332 ± 17	9.1 ± 1.4	2.8 ± 0.4	0.0001	0.008
3	0.01	240	331 ± 19	9.0 ± 1.5	2.8 ± 0.4	0.0007	0.08
4	0.1	240	331 ± 22	8.5 ± 1.2*	2.6 ± 0.3*	0.008	0.9
5	1	240	331 ± 17	8.5 ± 1.2*	2.6 ± 0.3*	0.08	8.7
6	10	240	330 ± 18	9.0 ± 1.3	2.7 ± 0.4	0.76	85.1
7	100	120	319 ± 19*	10.0 ± 1.6*	3.2 ± 0.4*	7.83	877.5

*Significantly different from group 1. IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline.

The number and size of GST-P-positive foci in rat livers at week 16 is summarized in Table 2. The total numbers of GST-P-positive foci per unit area in the livers in the groups administered 0.001–1 p.p.m. IQ did not differ from the control value (0 p.p.m. group), and no significant increases were observed in any size range of GST-P-positive foci in these groups. Significant increases in the total numbers of GST-P-positive foci per unit area in the liver were observed in the 10 and 100 p.p.m. groups compared with the control. The numbers of GST-P-positive foci composed of 2–4 cells and 5–10 cells in the 10 p.p.m. group and GST-P-positive foci of all sizes in the 100 p.p.m. group were significantly increased.

Formation of IQ-DNA adduct and 8-OHdG in liver DNA. Representative autoradiograms of IQ-DNA adducts in livers are shown in Figure 1. The levels of IQ-DNA adducts in the livers of the 0 and 0.001 p.p.m. IQ-treated groups were under the detectable limit at week 4 (Table 3). IQ-DNA adducts were detectable in the livers of rats administered 0.01 p.p.m. IQ, and adduct formation increased in a dose-dependent manner in groups administered higher doses of IQ. No significant differences in 8-OHdG levels were observed in the liver DNA between any of the groups administered IQ and the control group (Table 3).

Gene expression changes in the liver. Relative mRNA expression of IQ metabolizing genes CYP1A 1and CYP1A2, cell cycle genes PCNA and $p21^{Cip/WAF1}$, p53, and DNA repair genes APE-1 and GADD45 in the livers at week 16 is shown in Figure 2. CYP1A1 was significantly increased in the livers of rats treated with 100 p.p.m. IQ, but not in the lower doses of IQ. CYP1A2, on the other hand, was significantly increased in the 0.01–10 p.p.m. groups, but no significant change was observed in the 100 p.p.m. group. There was no significant difference in the CYP1B1 expression level among groups (data not shown).

A significant increase in PCNA was observed in the 100 p.p.m. group, but not in the groups administered lower doses of IQ, while the negative cell cycle regulator $p21^{Cip/WAF1}$ was significantly induced in the 0.01 p.p.m. group and maximally induced in the 100 p.p.m. group. The expression level of $p21^{Cip/WAF1}$ in the 100 p.p.m. group was significantly higher than in the 10 p.p.m. and lower dose groups. There were no significant changes in p53 expression levels in the IQ-treated groups.

APE-1 was significantly induced in the 10 and 100 p.p.m. groups and GADD45 was significantly induced in the 100 p.p.m. group. IQ had no effect on the expression of Ogg-1, MSH2 or MSH3 (data not shown).

Induction of ACF in the colon. The number and size of ACF in rat colons at week 16 is summarized in Table 4. In the 10 p.p.m. group, the number of ACF composed of one crypt was significantly increased compared with the control. In the 100 p.p.m. group, significant increases were observed in the numbers of all sizes of ACF. In contrast, in the groups administered 0.001–1 p.p.m. IQ, neither the number of any size ACF nor the total number of ACF differed from the control.

Discussion

Dose-response relationships for genotoxic carcinogens have been a topic of intense scientific and public debate. High doses of the genotoxic dietary carcinogen IQ have been demonstrated to induce liver and colon cancers in rats (300 p.p.m. in diet)[8] and liver cancers in nonhuman primates (10 mg/kg b.w./day).[10] However, as the concentrations of IQ in food are generally extremely low,[11] there is uncertainty regarding the carcinogenicity of the doses of IQ to which humans are exposed. The present study shows that IQ at doses of 1 p.p.m. (0.08 mg/kg body weight [b.w.]/day) and lower did not induce either GST-P-positive foci in the liver or ACF in the colon. Only in the groups administered higher doses of IQ, 10 p.p.m. (0.76 mg/kg b.w./day) and 100 p.p.m. (7.83 mg/kg b.w./day), were increases in GST-P-positive foci and ACF observed.

GST-P-positive foci and ACF are well-established preneoplastic lesions of the liver and colon, respectively, in rats. These lesions have been accepted as useful end-point markers in the assessment of carcinogenic effects of environmentally relevant concentrations of carcinogens as they can extend the range of observable effect levels.[24,26] Therefore, the results of the present study suggest the presence of no-effect levels of IQ for both liver and colon carcinogenicity in rats and indicate that the dose-response relationship for carcinogenicity of low dose IQ is nonlinear.

Several threshold mechanisms for genotoxic carcinogens have been suggested, including induction of detoxification processes, cell cycle delay, DNA repair, apoptosis and the suppression of neoplastically transformed cells by the immune system.[12,13,15,33] However, little *in vivo* evidence is available. To explore mechanisms underlying the carcinogenicity of low doses of IQ, we examined the relative mRNA expression of a panel of genes involved in cell proliferation, cell cycle regulation, DNA repair and IQ metabolic activation. We found that the cell proliferation marker PCNA was significantly increased only at a dose of 100 p.p.m., a dose that is carcinogenic. The cell cycle negative regulator $p21^{Cip/WAF1}$, on the other hand, was significantly induced at a dose of 0.01 p.p.m., a dose well below that which induced the formation of preneoplastic lesions. Furthermore, the finding that the levels of $p21^{Cip/WAF1}$ in the groups administered 10 p.p.m. and less were much lower than that of the group administered 100 p.p.m. implies that hepatocytes have adequate capacity to cope with the type of damage that is repaired by the $p21^{Cip/WAF1}$ pathway when exposed to low doses of IQ, but that the repair capacity of these hepatocytes, even in the presence of high $p21^{Cip/WAF1}$ expression, can be overwhelmed when the cell is subjected to very high doses of IQ. It is reasonable to suggest

Table 2. Development of GST-P-positive foci in the livers of rats administered IQ for 16 weeks

Group	IQ (p.p.m.)	No. rats	Size of GST-P positive foci				
			2–4	5–10	11–20	≥21	Total
1	0	240	0.09 ± 0.25	0.03 ± 0.11	0.02 ± 0.11	0.00 ± 0.02	0.15 ± 0.31
2	0.001	240	0.10 ± 0.24	0.04 ± 0.15	0.01 ± 0.07	0	0.16 ± 0.31
3	0.01	240	0.15 ± 0.47	0.07 ± 0.41	0.02 ± 0.22	0.02 ± 0.03	0.26 ± 1.30
4	0.1	240	0.10 ± 0.28	0.04 ± 0.15	0.01 ± 0.07	0.01 ± 0.08	0.15 ± 0.35
5	1	240	0.10 ± 0.25	0.04 ± 0.16	0.01 ± 0.06	0	0.14 ± 0.33
6	10	240	0.51 ± 0.65	0.19 ± 0.36*	0.02 ± 0.10	0.01 ± 0.11	0.74 ± 0.88*
7	100	120	26.23 ± 18.24*	23.81 ± 16.23*	19.25 ± 11.70*	18.74 ± 11.81*	88.03 ± 50.41*

*Significantly different from group 1. GST-P, glutathione *S*-transferase placental form positive foci; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline.

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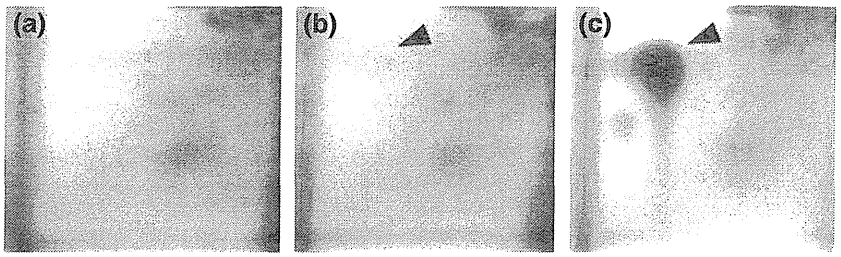

Fig. 1. Autoradiograms of 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ)-DNA adducts the livers of 0 (a), 0.001 (b) and 100 (c) p.p.m. IQ-treated groups at week 4. Arrowheads indicate IQ-DNA adduct. The imaging plates were exposed for 3 h (a) and 24 h (b and c).

Table 3. IQ-DNA adduct and 8-OHdG formation in liver DNA

Group	IQ (p.p.m.)	No. rats	Adduct level ($\times 10^{-7}$ ntd)	8-OHdG ($\times 10^{-5}$ dG)
1	0	5	UDL	0.23 ± 0.07
2	0.001	5	UDL	0.25 ± 0.05
3	0.01	5	0.045 ± 0.02	0.24 ± 0.07
4	0.1	5	0.1 ± 0.004	0.32 ± 0.10
5	1	5	1.7 ± 0.07	0.24 ± 0.08
6	10	5	12.7 ± 0.07	0.22 ± 0.07
7	100	5	107.0 ± 0.07	0.23 ± 0.08

IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; ntd, nucleotide; 8-OHdG: 8-hydroxy-2′-deoxyguanosine; UDL, under the detectable limit.

that suppression of cell cycle progression by $p21^{Cip/WAF1}$ followed by DNA repair is at least one of the mechanisms responsible for the observed no-effect of low doses of IQ in rats in the present model.

It is known that the vast majority of DNA damage is repaired by base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR).[34] APE-1 plays an essential role in the BER repair process by cleaving the phosphodiester backbone.[35] The activities of two different heterodimeric complexes, MSH2-MSH3 and MSH2-MSH6, belonging to the MMR system are mainly responsible for the post-replicative repair of mismatches.[36] We found that IQ significantly increased the expression levels of APE-1 but not MSH2 and MSH3 at doses of 10 and 100 p.p.m. in the liver. It has also been reported that IQ has no effect on expression of ERCC1, which is a key molecule in the NER process.[37] These findings suggest that BER rather than MMR or NER responds to IQ-induced DNA damage.

GADD45 is involved in a variety of growth regulatory mechanisms, including DNA repair, growth arrest and apoptosis.[38] It is induced by genotoxic and certain other cell stresses by p53-dependent and independent pathways.[39,40] GADD45 expression was significantly induced in the 100 p.p.m. group. The fact that significant induction of APE-1 and GADD45 was observed only at the highest doses of 10 and/or 100 p.p.m. indicate the IQ-induced DNA damage response is dose-dependent. Moreover, the fact that in the groups with low doses expression of APE-1 and GADD45 were not affected and that there was a significant but moderate induction of $p21^{Cip/WAF1}$ imply that normal physiological levels of these genes are sufficient to repair the DNA damage caused by low doses of IQ. However, the expression levels of these genes are all increased by higher carcinogenic doses of IQ. A reasonable explanation of the no-effect of low doses of IQ and the carcinogenicity of high doses of IQ is that carcinogenicity is the consequence of a disruption in the balance between DNA damage and repair and between abnormal cell proliferation and apoptosis or cell cycle regulation.

Our results show that p53 gene expression is not induced by administration of IQ. Furthermore, p53-deficient mice do not show higher susceptibility to IQ-induced liver carcinogenesis than wild type mice.[41] These results suggest that p53 does not have a significant impact on the carcinogenicity of IQ.

DNA adduct formation by metabolic activation of IQ is believed to play an important role in the carcinogenicity of IQ.[42] Formation of IQ-DNA adducts in the liver showed a linear dose-dependency and proved to be one of the most sensitive end-points for the detection of exposure to IQ. Adduct formation was detectable in groups administered far lower doses of IQ compared with detection of other end-points such as cell proliferation and preneoplastic lesion induction. That IQ-DNA adduct formation was not detected in the 0.001 p.p.m. group was most likely due to the detection limit of the assay. It should be noted that DNA adduct is a premutagenic lesion and not necessarily correlated to the frequencies of mutation and cancer induced by genotoxic compounds. For example, it is known that IQ forms DNA adducts in the kidneys and stomach of both rats and monkeys, but does not induce tumors in these organs.[43,44] Our present findings of a linear dose-response of IQ-DNA adduct formation and a nonlinear carcinogenic dose-response to IQ administration support the idea that IQ-DNA adducts do not necessarily lead to mutation and formation of cancerous lesions. Our results are also in line with previous results on HCA including MeIQx[1,18,45] and PhIP.[24] These results can be explained, at least in part, by the actions of gene products such as $p21^{Cip/WAF1}$, GADD45 and APE-1 and the other repair genes for DNA damage. Moreover, in the case of MeIQx, it has been suggested that formation of DNA adducts alone might not be sufficient to produce cancers and that the MeIQx-induced genetic alterations in the liver are enhanced by liver regeneration induced by high doses of MeIQx itself.[1] Therefore, while IQ-DNA adduct formation is important in IQ carcinogenicity, high levels of adduct formation are likely required and other factors such as cell proliferation can affect the balance between DNA damage and repair and lead to fixation of DNA mutations into the cell's genome.

It has been demonstrated *in vitro* that IQ is more efficiently metabolized and activated by CYP1A2 than by CYP1A1 or CYP1B1.[46] However, limited *in vivo* data are available. In a study by McPherson *et al.*[47], no significant induction in mRNA expression level or activity of either CYP1A1 or CYP1A2 were reported in the livers of rats receiving 300 p.p.m. IQ in the diet for 52 weeks, but these enzymes were significantly increased after daily administration of 20 mg/kg b.w. IQ by oral gavage for 3 days; in the average adult rat, a dose of 300 p.p.m. IQ in the diet is approximately equivalent to administration of 20 mg/kg b.w. IQ by oral gavage. The results of the present study revealed that IQ significantly induced CYP1A2 expression at doses from 0.01 to 10 p.p.m., but CYP1A2 was not induced in the 100 p.p.m. group. The lack of effect of 100 p.p.m. IQ on CYP1A2 expression is consistent with the results in rats receiving 300 p.p.m. IQ in the diet for 52 weeks.[47] Significant increases in CYP1A1 expression in the 100 p.p.m. group provide an alternative mechanism that can compensate for decreased CYP1A2 activity. However, as noted above, in apparent contrast to our results, in the study by McPherson *et al.*,[47] administration of 300 p.p.m. IQ over the course of 52 weeks did

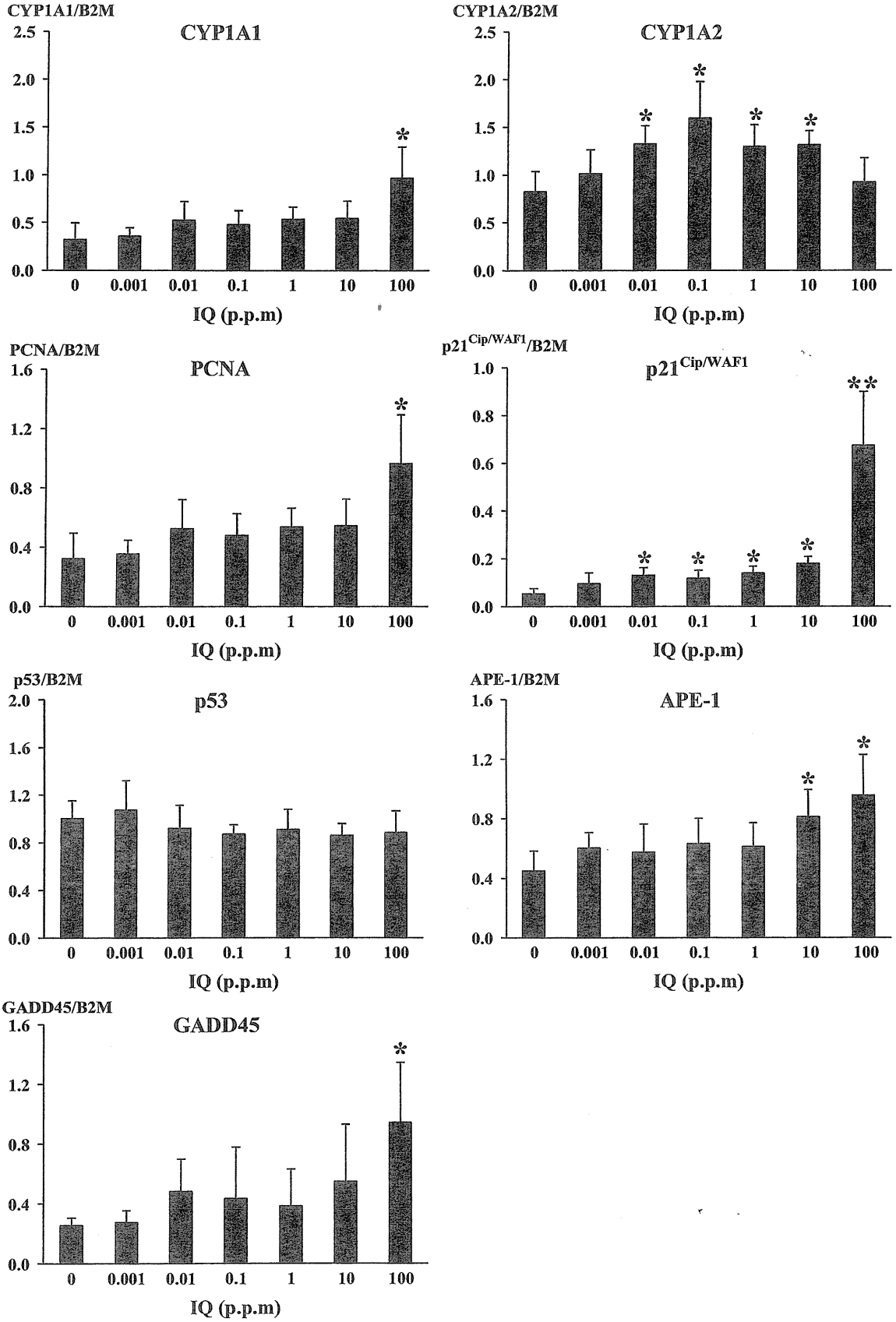

Fig. 2. Relative mRNA expression in the livers of rats at week 16. *Significantly different from 0 p.p.m. **Significantly different from all other groups. APE-1, AP endonuclease-1; B2M, beta-2-microglobulin; GADD45, growth arrest and DNA damage-inducible protein 45; PCNA, proliferating cell nuclear antigen.

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Table 4. Development of ACF in the colons of rats administered IQ for 16 weeks

Group	IQ (p.p.m.)	No. rats	Size of ACF				
			1	2	3	≥4	Total
1	0	240	0.08 ± 0.28	0.12 ± 0.32	0.06 ± 0.25	0.08 ± 0.29	0.33 ± 0.64
2	0.001	240	0.12 ± 0.36	0.08 ± 0.29	0.10 ± 0.32	0.09 ± 0.30	0.39 ± 0.69
3	0.01	240	0.15 ± 0.41	0.15 ± 0.42	0.06 ± 0.24	0.06 ± 0.24	0.43 ± 0.77
4	0.1	240	0.11 ± 0.33	0.11 ± 0.35	0.06 ± 0.25	0.08 ± 0.27	0.36 ± 0.63
5	1	240	0.15 ± 0.45	0.10 ± 0.30	0.10 ± 0.33	0.05 ± 0.23	0.41 ± 0.80
6	10	240	0.19 ± 0.48*	0.16 ± 0.41	0.07 ± 0.25	0.09 ± 0.40	0.50 ± 0.86
7	100	120	1.48 ± 1.46*	1.29 ± 1.51*	0.70 ± 0.93*	0.72 ± 1.01*	4.19 ± 3.34*

*Significantly different from group 1. ACF, aberrant crypt foci; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline.

not induce CYP1A1. Therefore, it is reasonable to postulate that the dose-relationship between IQ and induction of CYP1A1 is not a simple dose-response. CYP1B1 does not appear to be involved in the metabolism of IQ at doses up to 100 p.p.m. in rats. The findings described above demonstrate the importance of taking into account dosage, duration and route of exposure in interpretation of the data on metabolic activation of IQ. Further studies on the dose-response relationships between chronic IQ exposure and the protein expression levels and activities of detoxifying enzymes, especially at doses relevant to human exposure, would provide further insight into the role of metabolic activation in IQ carcinogenicity.

Oxidative DNA damage does not appear to play a role in IQ-induced carcinogenesis. In the present study, no significant changes in 8-OHdG levels or Ogg1 expression levels in the livers of IQ-treated rats were observed. Our results are consistent with the recent findings in IQ-treated Big Blue rats that oxidative stress was not responsible for the initiation of IQ-induced carcinogenesis in the liver and colon.[37] In this respect, IQ is different from MeIQx, in which oxidative DNA damage plays an important role in liver carcinogenesis.[48]

In summary, the present study provides the first experimental data on the carcinogenicity of low doses of IQ in both the liver and colon of the test animal and compares the effect of IQ at the cellular level with its carcinogenic effect. Our findings support the idea that there is a practical threshold that should be considered when evaluating the risk of genotoxic carcinogens. To this end, further accumulation of data, especially mechanistic data, should be promoted to facilitate not only an understanding of the carcinogenic effects of low doses of genotoxic carcinogens but also to establish an accurate means of quantitative risk assessment.

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Disclosure Statement

The authors have no conflict of interest.

References

1 Wakabayashi K, Nagao M, Esumi H, Sugimura T. Food-derived mutagens and carcinogens. *Cancer Res* 1992; **52**: 2092s–8s.
2 Layton DW, Bogen KT, Knize MG, Hatch FT, Johnson VM, Felton JS. Cancer risk of heterocyclic amines in cooked foods: an analysis and implications for research. *Carcinogenesis* 1995; **16**: 39–52.
3 Nagao M, Ushijima T, Toyota M, Inoue R, Sugimura T. Genetic changes induced by heterocyclic amines. *Mutat Res* 1997; **376**: 161–7.
4 Kato T, Ohgaki H, Hasegawa H, Sato S, Takayama S, Sugimura T. Carcinogenicity in rats of a mutagenic compound, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline. *Carcinogenesis* 1988; **9**: 71–3.
5 Ohgaki H, Hasegawa H, Suenaga M, Sato S, Takayama S, Sugimura T. Carcinogenicity in mice of a mutagenic compound, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) from cooked foods. *Carcinogenesis* 1987; **8**: 665–8.
6 Ito N, Hasegawa R, Sano M *et al.* A new colon and mammary carcinogen in cooked food, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Carcinogenesis* 1991; **12**: 1503–6.
7 Esumi H, Ohgaki H, Kohzen E, Takayama S, Sugimura T. Induction of lymphoma in CDF1 mice by the food mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Jpn J Cancer Res* 1989; **80**: 1176–8.
8 Ohgaki H, Hasegawa H, Kato T *et al.* Carcinogenicity in mice and rats of heterocyclic amines in cooked foods. *Environ Health Perspect* 1986; **67**: 129–34.
9 Ohgaki H, Kusama K, Matsukura N *et al.* Carcinogenicity in mice of a mutagenic compound, 2-amino-3-methylimidazo[4,5-f]quinoline, from broiled sardine, cooked beef and beef extract. *Carcinogenesis* 1984; **5**: 921–4.
10 Adamson RH, Thorgeirsson UP, Snyderwine EG *et al.* Carcinogenicity of 2-amino-3-methylimidazo[4,5-f]quinoline in nonhuman primates: induction of tumors in three macaques. *Jpn J Cancer Res* 1990; **81**: 10–4.
11 WHO, IARC. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Nuturally Occurring Substances: Food Items and Constituents, Heterocylic Aromatic Amines and Mycotoxins*. Lyon: World Health Organization, International Agency for Research on Cancer, 1993; 165–242.
12 Sofuni T, Hayashi M, Nohmi T, Matsuoka A, Yamada M, Kamata E. Semi-quantitative evaluation of genotoxic activity of chemical substances and evidence for a biological threshold of genotoxic activity. *Mutat Res* 2000; **464**: 97–104.
13 De Flora S. Detoxification of genotoxic compounds as a threshold mechanism limiting their carcinogenicity. *Toxicol Pathol* 1984; **12**: 337–43.
14 Kirkland DJ, Muller L. Interpretation of the biological relevance of genotoxicity test results: the importance of thresholds. *Mutat Res* 2000; **464**: 137–47.
15 Lutz WK, Kopp-Schneider A. Threshold dose response for tumor induction by genotoxic carcinogens modeled via cell-cycle delay. *Toxicol Sci* 1999; **49**: 110–5.
16 Parry JM. Reflections on the implications of thresholds of mutagenic activity for the labelling of chemicals by the European Union. *Mutat Res* 2000; **464**: 155–8.
17 Hoshi M, Morimura K, Wanibuchi H *et al.* No-observed effect levels for carcinogenicity and for in vivo mutagenicity of a genotoxic carcinogen. *Toxicol Sci* 2004; **81**: 273–9.
18 Fukushima S, Wanibuchi H, Morimura K *et al.* Lack of a dose-response relationship for carcinogenicity in the rat liver with low doses of 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline or N-nitrosodiethylamine. *Jpn J Cancer Res* 2002; **93**: 1076–82.

19 Bolt HM, Degen GH. Human carcinogenic risk evaluation, part II: contributions of the EUROTOX specialty section for carcinogenesis. *Toxicol Sci* 2004; **81**: 3–6.
20 Fukushima S, Wanibuchi H, Morimura K *et al.* Lack of initiation activity in rat liver of low doses of 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline. *Cancer Lett* 2003; **191**: 35–40.
21 Kushida M, Wanibuchi H, Morimura K *et al.* Dose-dependence of promotion of 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline-induced rat hepatocarcinogenesis by ethanol: evidence for a threshold. *Cancer Sci* 2005; **96**: 747–57.
22 Wei M, Hori TA, Ichihara T *et al.* Existence of no-observed effect levels for 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline on hepatic preneoplastic lesion development in BN rats. *Cancer Lett* 2006; **231**: 304–8.
23 Doi K, Wanibuchi H, Salim EI *et al.* Lack of large intestinal carcinogenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine at low doses in rats initiated with azoxymethane. *Int J Cancer* 2005; **115**: 870–8.
24 Fukushima S, Wanibuchi H, Morimura K *et al.* Existence of a threshold for induction of aberrant crypt foci in the rat colon with low doses of 2-amino-1-methyl-6-phenolimidazo[4,5-b]pyridine. *Toxicol Sci* 2004; **80**: 109–14.
25 Ito N, Tsuda H, Tatematsu M *et al.* Enhancing effect of various hepatocarcinogens on induction of preneoplastic glutathione S-transferase placental form positive foci in rats – an approach for a new medium-term bioassay system. *Carcinogenesis* 1988; **9**: 387–94.
26 Tsuda H, Fukushima S, Wanibuchi H *et al.* Value of GST-P positive preneoplastic hepatic foci in dose-response studies of hepatocarcinogenesis: evidence for practical thresholds with both genotoxic and nongenotoxic carcinogens. A review of recent work. *Toxicol Pathol* 2003; **31**: 80–6.
27 Bird RP. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett* 1987; **37**: 147–51.
28 Tudek B, Bird RP, Bruce WR. Foci of aberrant crypts in the colons of mice and rats exposed to carcinogens associated with foods. *Cancer Res* 1989; **49**: 1236–40.
29 Ochiai M, Nakagama H, Turesky RJ, Sugimura T, Nagao M. A new modification of the 32P-post-labeling method to recover IQ-DNA adducts as mononucleotides. *Mutagenesis* 1999; **14**: 239–42.
30 Totsuka Y, Fukutome K, Takahashi M *et al.* Presence of N2-(deoxyguanosin-8-yl)-2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (dG-C8-MeIQx) in human tissues. *Carcinogenesis* 1996; **17**: 1029–34.
31 Wei M, Hamoud AS, Yamaguchi T *et al.* Potassium bromate enhances N-ethyl-N-hydroxyethylnitrosamine-induced kidney carcinogenesis only at high doses in Wistar rats: indication of the existence of an enhancement threshold. *Toxicol Pathol* 2009; **37**: 983–91.
32 Pretlow TP, O'Riordan MA, Somich GA, Amini SB, Pretlow TG. Aberrant crypts correlate with tumor incidence in F344 rats treated with azoxymethane and phytate. *Carcinogenesis* 1992; **13**: 1509–12.
33 Kirsch-Volders M, Vanhauwaert A, Eichenlaub-Ritter U, Decordier I. Indirect mechanisms of genotoxicity. *Toxicol Lett* 2003; **140–141**: 63–74.
34 Norbury CJ, Hickson ID. Cellular responses to DNA damage. *Annu Rev Pharmacol Toxicol* 2001; **41**: 367–401.
35 Bennett RA, Wilson DM III, Wong D, Demple B. Interaction of human apurinic endonuclease and DNA polymerase beta in the base excision repair pathway. *Proc Natl Acad Sci U S A* 1997; **94**: 7166–9.
36 Kolodner RD. Guarding against mutation. *Nature* 2000; **407**: 687–9.
37 Moller P, Wallin H, Vogel U *et al.* Mutagenicity of 2-amino-3-methylimidazo[4,5-f]quinoline in colon and liver of Big Blue rats: role of DNA adducts, strand breaks, DNA repair and oxidative stress. *Carcinogenesis* 2002; **23**: 1379–85.
38 Yang Q, Manicone A, Coursen JD *et al.* Identification of a functional domain in a GADD45-mediated G2/M checkpoint. *J Biol Chem* 2000; **275**: 36892–8.
39 O'Reilly MA, Staversky RJ, Watkins RH, Maniscalco WM, Keng PC. p53-independent induction of GADD45 and GADD153 in mouse lungs exposed to hyperoxia. *Am J Physiol Lung Cell Mol Physiol* 2000; **278**: L552–9.
40 Shaulian E, Karin M. Stress-induced JNK activation is independent of Gadd45 induction. *J Biol Chem* 1999; **274**: 29595–8.
41 Morimura K, Salim EI, Yamamoto S, Wanibuchi H, Fukushima S. Dose-dependent induction of aberrant crypt foci in the colons but no neoplastic lesions in the livers of heterozygous p53-deficient mice treated with low dose 2-amino-3-methylimidazo [4,5-f]quinoline. *Cancer Lett* 1999; **138**: 81–5.
42 Schut HA, Snyderwine EG. DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis. *Carcinogenesis* 1999; **20**: 353–68.
43 Schut HA, Herzog CR, Cummings DA. Accumulation of DNA adducts of 2-amino-3-methylimidazo[4,5-f] quinoline (IQ) in tissues and white blood cells of the Fischer-344 rat after multiple oral dosing. *Carcinogenesis* 1994; **15**: 1467–70.
44 Snyderwine EG, Yamashita K, Adamson RH *et al.* Use of the 32P-postlabeling method to detect DNA adducts of 2-amino-3-methylimidazolo[4,5-f]quinoline (IQ) in monkeys fed IQ: identification of the N-(deoxyguanosin-8-yl)-IQ adduct. *Carcinogenesis* 1988; **9**: 1739–43.
45 Yamashita K, Adachi M, Kato S *et al.* DNA adducts formed by 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline in rat liver: dose-response on chronic administration. *Jpn J Cancer Res* 1990; **81**: 470–6.
46 Shimada T, Hayes CL, Yamazaki H *et al.* Activation of chemically diverse procarcinogens by human cytochrome P-450 1B1. *Cancer Res* 1996; **56**: 2979–84.
47 McPherson RA, Tingle MD, Ferguson LR. Contrasting effects of acute and chronic dietary exposure to 2-amino-3-methyl-imidazo[4,5-f]quinoline (IQ) on xenobiotic metabolising enzymes in the male Fischer 344 rat: implications for chemoprevention studies. *Eur J Nutr* 2001; **40**: 39–47.
48 Kato T, Hasegawa R, Nakae D *et al.* Dose-dependent induction of 8-hydroxyguanine and preneoplastic foci in rat liver by a food-derived carcinogen, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline, at low dose levels. *Jpn J Cancer Res* 1996; **87**: 127–33.

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Non-genotoxic mode of action and possible threshold for hepatocarcinogenicity of Kojic acid in F344 rats

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ABSTRACT

Kojic acid (KA), a naturally occurring compound, is contained in traditional Japanese fermented foods and is used as a food additive, preservative and a dermatological skin-lightening agent. In the present experiment, initiation (experiment 1) and promotion (experiment 2) effects of KA-induced hepatocarcinogenesis were studied by rat medium-term bioassay for carcinogenicity. Male F344 rats were administered a diet containing 0–2% KA. Experiment 1 demonstrated that KA had no effect on induction of liver preneoplastic lesions or glutathione *S*-transferase placental form (GST-P) positive foci, in either number or area. In experiment 2, 2% KA treatment significantly increased the number and area of GST-P positive foci, but concentrations less than 0.5% did not. Moreover, 2% KA treatment significantly increased 8-OHdG levels and PCNA positive hepatocytes. The results indicated that low concentrations of KA do not have initiation effects on rat hepatocarcinogenesis, while higher concentrations of KA do promote hepatocarcinogenesis in rats. Thus, the results indicate that KA is a non-genotoxic hepatocarcinogen, showing the possible existence of a perfect threshold.

1. Introduction

Chemical carcinogenesis, including hepatocarcinogenesis, has been classified into 'initiation' and 'promotion' stages. At present, promotion has further been subdivided into 'promotion' and 'progression'. Initiation involves mutation of hepatocellular DNA, thought to be irreversible and to consist of a single gene mutation that is caused by environmental genotoxic chemical agents, both physical and biological (e.g., viruses). Promotion follows initiation and involves aspects of gene activation, such as the latent phenotype of the initiated cell, which becomes expressed through cellular selection and clonal growth. This can proceed through a variety of mechanisms, including failure of apoptosis and failure to terminate proliferation of the initiated cell. Progression, the last stage, involves genetic damage that results in the conversion of benign tumors into malignant neoplasms capable of invading adjacent tissues and metastasizing to distant sites. The neoplastic progression may happen due to the errors in DNA replication and repairing DNA damage.

The various tests that have been used to identify carcinogenic agents may be classified into short, medium and long-term bioassays. Short-term assays have limited use, indirectly establishing an estimate of the risk that such chemicals pose for the human population. Long-term assays, for detection of carcinogenicity in experimental animals, have been the foundation for prediction of carcinogenic hazards to human beings. The standard requirements are – lifetime carcinogenicity testing in two rodent species, rats and mice, at three different doses of test compound with controls for each sex. This is, however, time-consuming and very expensive in terms of financial and human resources. Good animal facilities with experts in toxicology and toxicological pathology are essential for performance of the assays. Evaluating the performance of long-term bioassays, it became obvious that medium-term bioassays for carcinogenicity should be designed so as to reduce the time for the development of an endpoint. Carcinogens are generally classified into two categories: genotoxic and non-genotoxic. Genotoxic carcinogens have both initiation activity and promotion activity, and non-genotoxic carcinogens have promotion activity without initiation activity. Based on this concept, two-stage carcinogenic models as a medium-term carcinogenicity test have been developed for the detection of carcinogenicity in chemicals. Concerning the hepatocarcinogenicity of chemicals, medium-term carcinogenicity tests targeted for initiation activity or promotion activity have been established. The medium-term hepatocarcinogenesis bioassay of Ito et al. (1989, 2003), an 8-week experiment, utilizes

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F344 rats that are given a single dose of diethylnitrosamine (DEN) to initiate carcinogenesis. After a 2-week period the rats are repeatedly exposed to a test compound for a dosing period of 6 weeks. At the end of week 3, rats are subjected to partial hepatectomy to maximize opportunities of promotion via a high rate of cell proliferation. All rats are sacrificed at the end of week 8 for evaluation of the development of preneoplastic hepatocellular foci by staining for expression of glutathione *S*-transferase placental form (GST-P) (Ito et al., 1989, 2003). Extensive testing has demonstrated that the induction of GST-P positive foci in the medium-term bioassay for liver carcinogens correlates well with the incidence of hepatocellular carcinomas in parallel long-term assays.

Genotoxic carcinogens are mutagenic and seem to act through interaction with DNA to produce irreversible genetic changes in target organ cells. It has been generally concluded that they have no dose threshold in their carcinogenic potential. Dose response assessment, to define relationships between doses of agents and the probability of induction of carcinogenic effect, is one of the most important components of risk assessment to humans. The dose–response relationship for genotoxic carcinogens is generally assumed to be linear without a threshold dose below which carcinogenic effects are absent. This means that genotoxic carcinogens may pose some risk at any level of exposure, although there is no definitive experimental evidence to support this suggestion. Several recently published papers attempt to find experimental evidence for the existence of a threshold in the dose–response for genotoxic carcinogens.

The different types of thresholds for carcinogens are opposed to the classical dose–response of carcinogens for which no threshold can be defined (Bolt et al., 2004). Hengstler et al. (2003) distinguished between perfect and practical thresholds, based on different types of mechanisms. Basically, non-genotoxic carcinogens have been connected with the perfect threshold. The carcinogens do not produce any DNA damage through inability of mode of action. A 'perfect threshold' has been attributed to mitotic spindle poisons where the primary interaction occurs with proteins and not with DNA. The definition of 'practical threshold' is based on the concept that the carcinogens should have no genotoxic effect at very low or immeasurable target concentrations. Such practical thresholds have been connected with rapid degradation (toxicokinetics) of the chemical or to factors in general that limit target exposures (Kirsch-Volders et al., 2000).

The human diet contains substantial amounts of a wide variety of natural mutagens and carcinogens. Kojic acid (KA; 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one), a metabolic product of several species of *Aspergillus*, is contained in traditional Japanese fermented foods, including miso (soybean paste), soy sauce and sake, in levels up to 1 ppm (Burdock et al., 2001) and used as a food additive and preservative, and as a skin-whitening agent in cosmetics.

KA has an obscure toxicological profile. It was reported to be genotoxic in several *in vitro* tests, including mutation in *Salmonella typhimurium* strains TA98, TA100 and TA1535 either with or without metabolic activation, and the sister chromatid exchanges and chromosome aberrations test in Chinese hamster ovary (CHO) cells (Wei et al., 1991). However, all of the *in vivo* genotoxicity tests on KA were negative, including two independent mouse bone marrow micronucleus tests after a single administration and an unscheduled DNA synthesis test in rat hepatocytes (Nohynek et al., 2004).

It has been demonstrated that hepatocellular tumors were induced in B6C3F1 mice that were fed a diet containing 3% KA for 20 months (Fujimoto et al., 1998). In another study, it was reported that the incidence of hepatocellular adenomas as well as altered hepatocellular foci were increased in *p53*(+/−) and *p53*(+/+) mice fed 1.5% or 3% KA, as compared to those in untreated control mice (Takizawa et al., 2003). A recent report showed that a 20-week dietary administration of 2% KA with initiation treatment of *n*-bis(2-hydroxypropyl) nitrosamine (DHPN) increased the number and area of GST-P in the liver of F344 rats (Takizawa et al., 2004) and suggested possible hepatocarcinogenic potential of KA in the rat. Moreover, the initiating activities of KA on hepatocarcinogenesis have been investigated in F344 rats. The rats were given a single dose of KA followed by dietary administration of 0.015% 2-acetylaminofluorene (2-AAF) for 2 weeks and a single dose of CCl_4. The results suggest that KA has neither liver initiation activity nor capability of oxidative stress formation, but some evidence suggestive of liver tumor promoting effects in rats (Watanabe et al., 2005). The tumor-initiating activity of KA using partially hepatectomized mice was investigated, and the results suggest that KA has no tumor-initiating activity in mice livers (Moto et al., 2006). Thus there is a high possibility that KA has no *in vivo* genotoxic effects in rats.

In addition, KA has been reported to induce thyroid adenomas and hyperplasia formation in rodents, and the result was suggested to be due to its promoting activity (Fujimoto et al., 1998; Mitsumori et al., 1999; Tamura et al., 2001). Epidemiological studies show the occurrence of low incidence of thyroid cancer in the Japanese population (Marugame et al., 2006).

To confirm indistinct hepatocarcinogenicity of KA, a modified Tsuda's method (Tsuda et al., 1990, 1993; Takada et al., 1997) has been used for examination of initiation activity of rat hepatocarcinogenesis, and Ito's model (Ito et al., 1989, 2003) was used for examination of promotion activity.

2. Materials and methods

2.1. Chemicals

KA (CAS No. 501-30-4) (purity ⩾ 98%) and DEN were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). KA was mixed into a powdered basal diet (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) at doses of 0.1%, 0.5% and 2% for experiment 1 (to examine initiation activity) and at doses of 0.001%, 0.01%, 0.1%, 0.5% and 2% for experiment 2 (to examine promotion activity). 2-AAF (CAS No. 53-96-3) was from Nacalai Tesque Inc., and mixed into powdered basal diet at a dose of 0.01%.

2.2. Animals

Five-week-old male F344 rats (60 rats in experiment 1 and 90 rats in experiment 2) were purchased from Charles River, Japan (Atsugi, Kanagawa, Japan). They were housed in an animal room, maintained on a 12 h (08:00–20:00) light/dark cycle at a constant temperature of 23 ± 1 °C and relative humidity of 44 ± 5% and were given free access to tap water and food. Animals were acclimated 1 week before the start of the experiment.

2.3. Experimental protocol

The scheme of the experiments is illustrated in Fig. 1. For detection of initiation activity of KA (experiment 1: liver initiation assay), 4 groups of 15 rats each were fed a diet containing 0, 0.1%, 0.5% or 2% KA for 4 weeks. Following an additional 2 weeks on a basal diet, rats were then fed a diet containing 0.01% 2-AAF for 2 weeks coupled with two-thirds partial hepatectomy during 2-AAF administration and continuation for an additional week's basal diet (Takada et al., 1997). The total observation period was 9 weeks.

For detection of the promotion activity of KA (experiment 2: liver promotion assay), the rats were divided into six groups (15 rats each). After 1 week on basal diet they underwent i.p. injection of DEN (200 mg/kg body wt.) dissolved in saline, to initiate