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Association of functional *FEN1* genetic variants and haplotypes and breast cancer risk☆

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ABSTRACT

Aim: As a tumor suppressor, FEN1 plays an essential role in preventing tumorigenesis. Two functional germline variants (-69G>A and 4150G>T) in the *FEN1* gene have been associated with DNA damage levels in coke-oven workers and multiple cancer risk in general populations. However, it is still unknown how these genetic variants are involved in breast cancer susceptibility.

Methods: We investigated the association between these polymorphisms and breast cancer risk in two independent case–control sets consisted of a total of 1100 breast cancer cases and 1400 controls. The influence of these variations on *FEN1* expression was also examined using breast normal tissues.

Results: It was found that the *FEN1*-69GG genotypes were significantly correlated to increased risk for developing breast cancer compared with the -69AA genotype in both sets [Jinan set: odds ratios (OR) = 1.41, 95% confidence interval (CI) = 1.20–1.65, $P = 1.9\times10^{-5}$; Huaian set: OR = 1.51, 95% CI = 1.22–1.86, $P = 1.7\times10^{-4}$]. Similar results were observed for 4150G>T polymorphism. The genotype–phenotype correlation analyses demonstrated that the -69G or 4150G allele carriers had more than 2-fold decreased *FEN1* expression in breast tissues compared with -69A or 4150T carriers, suggesting that lower *FEN1* expression may lead to higher risk for malignant transformation of breast cells.

Conclusion: Our findings highlight *FEN1* as an important gene in human breast carcinogenesis and genetic variants in *FEN1* confer susceptibility to breast cancer.

1. Introduction

Flap endonuclease 1 (FEN1) belongs to a kind of structure-specific nucleases and plays an essential part in efficient 5′-flap removal during the maturation of Okazaki fragments in DNA replication and long-patch base-excision repair (Harrington et al., 1994; Lieber, 1997; Shen et al., 2005). As a multiple-functional enzyme, FEN1 is also involved in a 5′ exonuclease (Balakrishnan et al., 2013) and a gap-dependent endonuclease (Reagan et al., 1995; Zheng et al., 2005). Due to its important role in DNA repair and other multiple DNA metabolic pathways, FEN1 has been considered as a key actor during maintenance of genomic stability and protection against tumorigenesis (Balakrishnan et al., 2013; Harrington et al., 1994; Lieber, 1997; Reagan et al., 1995; Shen et al., 2005; Zheng et al., 2005). It was showed that the functional damage of yeast RAD27 (the homolog of mammalian FEN1) results in an extraordinary increase in the rate of spontaneous mutations (Parrish et al., 2003; Tishkoff et al., 1997; Zheng et al., 2005). Haploinsufficient *FEN1* can lead to genome instability and carcinogenesis in mice (Kucherlapati et al., 2002). It has also been reported that *FEN1* mutations, which were identified in human cancer cells, results in reduced nuclease activity and 70% of mice knocked-in the mutated *FEN1* developed malignancies in multiple organs (Zheng et al., 2007). Therefore, aberrant expression of FEN1 resulting from naturally occurring genetic variants may contribute to cancer susceptibility.

After thoroughly re-sequencing the *FEN1* locus in 30 Chinese Han healthy volunteers, we identified two single nucleotide polymorphisms (SNP), -69G>A (rs174538, in the gene promoter region) and 4150G>T (rs4246215, in gene 3′-UTR) (Yang et al., 2009). It

Abbreviations: FEN1, Flap endonuclease 1; SNP, single nucleotide polymorphisms; RFLP, restriction fragment length polymorphism; ORs, odds ratios; 95% CIs, 95% confidence intervals.

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has been found that -69G>A SNP causes increased promoter activity, which is most likely to be due to a higher binding affinity of the G allele with some unknown transcriptional inhibitors. In addition, the 4150G>T SNP is also associated with differential levels of *FEN1* RNA expression. After examining *FEN1* RNA in lung tissues, we found that both -69G>A and 4150G>T SNPs could impact gene expression in vivo. These SNPs were reproducibly associated with risk of lung cancer, hepatocellular carcinoma, esophageal cancer, gastric cancer, colorectal cancer and glioma from different medical centers of China (Chen et al., 2013a; Liu et al., 2012).

However, their involvement in breast cancer development and impact on *FEN1* expression in breast tissues are still unclear. Therefore, we examined whether these two genetic variations contribute to susceptibility of breast cancer in two independent case–control sets from Jinan city (Shandong Province) and Huaian city (Jiangsu Province). The genotype–phenotype correlation study on the biological function of these two SNPs was also examined through detecting *FEN1* mRNA expression levels in breast tissues.

2. Materials and methods

2.1. Study case–control sets

There were two case–control sets in the current study. (i) Jinan case–control set: There are 800 cases with breast cancer from Shandong Cancer Hospital, Shandong Academy of Medical Sciences (Jinan, Shandong Province, China) and 800 sex- and age-matched (±5 years) healthy controls. The patients were recruited between June 2009 and April 2012 at Shandong Cancer Hospital. The controls were randomly selected from a pool of 4500 individuals from a community cancer-screening program for early detection of cancer conducted in Jinan city during the same time period as the patients were collected. (ii) Huaian case–control set contains 300 breast cancer cases from Huaian No. 2 Hospital (Huaian, Jiangsu Province, China) and 600 sex- and age-matched healthy controls. The patients were consecutively recruited between January 2009 and February 2012 at Huaian No. 2 Hospital. The control subjects were cancer-free individuals selected from a community cancer-screening program (3600 individuals) for early detection of cancer conducted in Huaian city during the same time period as the patients were collected. The diagnosis of all patients was histologically confirmed. Twenty-five breast normal tissues adjacent to the tumors were obtained from surgically removed specimens of patients in Huaian No. 2 Hospital. All the subjects were ethnic Han Chinese. This study was approved by the Institutional Review Boards and written informed consent was obtained from each individual at recruitment.

2.2. Polymorphism genotyping

FEN1 -69G>A and 4150G>T genotypes were genotyped using PCR-based restriction fragment length polymorphism (RFLP) as previously reported (Chen et al., 2013a; Liu et al., 2012; Yang et al., 2009). In PCR-RFLP genotyping, the primers used for amplifying DNA segments containing either the *FEN1* -69G>A or 4150G>T polymorphisms were 5′-ggaggttccaggagcgtcta-3′/5′-ttctccaccgcttgtccc-3′ or 5′-tatgtcaggctc aaaccac-3′/5′-cagccagtaatcagtcacaa-3′, respectively. Restriction enzymes *Sal*I (New England Biolabs) or *Alw*26I (Fermentas) was used to distinguish the -69G>A or 4150G>T genotypes, respectively. A 15% random sample was reciprocally tested by different persons, and the reproducibility was 99.8%. In addition, a 5% random sample was also examined by Sanger sequencing, and the reproducibility was 100%.

2.3. Real-time PCR analysis of FEN1 mRNA

Total cellular RNA was extracted from twenty-five breast tissue samples using TRIzol Reagent (Invitrogen). SYBR-Green real-time quantitative PCR method was used to examine *FEN1* mRNA levels in normal breast tissues as described previously (Liu et al., 2012; Zhang et al., 2013; Zhou et al., 2013). In brief, total cellular RNA was isolated and converted to cDNA using the ReverTra Ace qPCR RT Kit (TOYOBO). Relative gene expression quantitation for *FEN1* and *β-actin* as an internal reference gene was carried out using Quantitect SYBR Green Realtime PCR Master Mix (TOYOBO) with the ABI 7500 real-time PCR system in triplicates. The primers used for *FEN1* were 5′-ctgtggacctcatccagaagca-3′ and 5′-ccagcacctcaggttccaaga-3′; and for *β-actin* were 5′-ggcgg caccaccatgtaccct-3′ and 5′-aggggccggactcgtcatact-3′. The expression of individual *FEN1* measurements was calculated relative to expression of *β-actin* using the method as described previously (Lehmann et al., 2001; Zhang et al., 2010).

2.4. Statistical analyses

Pearson's χ^2 test was used to examine the differences in demographic variables and genotype distributions of *FEN1* polymorphisms between breast cancer patients and controls. The association between *FEN1* genotypes and risk of breast cancer was estimated by odds ratios (ORs) and their 95% confidence intervals (95% CIs) computed by logistic regression models. Student's *t* test was used to assess differences in *FEN1* transcript abundance with different genotypes. All ORs were adjusted for age and family history of breast cancer, where it was appropriate. A *P* value of less than 0.05 was used as the criterion of statistical significance, and all statistical tests were two-sided. All analyses were performed using SPSS software package (Version 16.0, SPSS Inc., Chicago, IL). Haploview 3.2 software was used to construct the haplotypes and Haplo.stats software package developed using the R language was used to estimate adjusted ORs and 95% CIs for each haplotype (Schaid et al., 2002). Simulations were run for 1000 times for empirical *P* values.

3. Results

Basic characteristics of the 1100 breast cancer patients and 1400 healthy controls included in the study are presented in Table 1. There are no statistically significant differences between cases and controls for the Jinan case–control set and Huaian case–control set in terms of median age distribution (both $P > 0.05$), indicating that the frequency matching was adequate (Table 1). In both case–control sets, there were no significant differences between patients and controls considering age at menarche, menstrual history and family history of breast cancer (all $P > 0.05$).

All observed genotype frequencies in both controls and patients conform to Hardy–Weinberg equilibrium. The allelic frequencies for the -69A and 4150T were 0.384 and 0.389 among 800 healthy controls in the Jinan set, and 0.430 and 0.434 among 600 control subjects in the Huaian set. The distributions of allelic frequencies of both SNPs were significantly different between Jinan and Huaian Chinese populations ($P < 0.05$). Linkage disequilibrium analysis showed that these two SNPs are in strong linkage, with $D' = 0.94$ and $r^2 = 0.81$ in the Jinan set and $D' = 0.90$ and $r^2 = 0.92$ in the Huaian set.

In either the Jinan set or Huaian set, carriers of the *FEN1* -69GG genotype have shown significantly and consistently elevated risks to develop breast cancer compared with -69AA carriers ($OR_{Jinan} = 1.41$, 95% CI = 1.20–1.65, $P = 1.9\times10^{-5}$, $OR_{Huaian} = 1.51$, 95% CI = 1.22–1.86, $P = 1.7\times10^{-4}$) (Table 2). Logistic regression analyses also revealed that individuals with the *FEN1* -69GA genotype were significantly associated with increased breast cancer risk in the Jinan set (OR = 1.41, 95% CI = 1.03–1.94, $P = 0.032$) (Table 2). However, no significant association between the -69GA genotype and breast cancer risk was observed in the Huaian set (OR = 1.32, 95% CI = 0.85–2.05, $P = 0.219$) (Table 2). In pooled data analyses, either the -69GA or -69GG genotype was significantly associated with increased breast cancer risk (both $P < 0.01$) (Table 2).

For the *FEN1* 4150G>T polymorphism, we found that the 4150GG genotype had a 1.22-fold or 1.62-fold increased risk for breast cancer