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Postprint: Interaction of PPAR- 2 C34G and NADPH Oxidase p22phox C242T Polymorphisms with Helicobacter pylori Infection in Esophageal Squamous Cell Carcinoma

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Abstract

Objective: To investigate the interaction between peroxisome proliferator-activated receptor- 2 (PPAR- 2) gene -C34G polymorphism, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase p22phox subunit gene -C242T polymorphism and Helicobacter pylori (H. pylori) infection, and their relationship with esophageal squamous cell carcinoma (ESCC). Methods: Patients with ESCC Broder grade I, Broder grade II, and Broder grade III (200 cases each) admitted to our hospital from May 2010 to March 2015 were selected, with 200 healthy individuals serving as the control group. Using peripheral blood leukocytes from the aforementioned groups as samples, PCR-RFLP was utilized to detect PPAR- 2 gene -C34G and NADPH oxidase p22phox subunit gene -C242T polymorphisms. 14C-UBT was employed to detect the disintegrations per minute (DPM) of 14C binding with H. pylori in examinees to determine H. pylori infection status. Unconditional logistic regression was used to analyze the interaction between -C34G, -C242T polymorphisms and H. pylori infection. Results: Individuals with -C34G (CG) and -C34G (GG) genotypes exhibited significantly increased risk of ESCC, and those with -C242T (CT) and -C242T (TT) genotypes also showed significantly increased risk of ESCC. Synergistic analysis of gene mutations revealed that -C34G (GG) and -C242T (TT) genotypes had a positive interaction in the occurrence and progression of ESCC. Additionally, positive interactions existed between -C34G (CG) and -C242T (TT), between -C34G (CG) and -C242T (CT), and between -C34G (GG) and -C242T (CT) (all >1). The risk of ESCC was significantly elevated in H. pylori-infected individuals. H. pylori infection had positive interactions with -C34G (CG), -C34G (GG), -C242T (CT) and -C242T (TT) genotypes (all >1). Conclusion: Individuals carrying -C34G (CG), -C34G (GG), -C242T (CT) and -C242T (TT) genotypes constitute

high-risk populations for ESCC. The interaction between these genotypes and H. pylori infection promotes the occurrence and progression of ESCC. Measures to eradicate H. pylori or regulate gene expression should be implemented for effective prevention of LSCC.

Full Text

Interaction of Polymorphisms of PPAR- 2 Gene -C34G and NADPH Oxidase Subunit p22phox Gene -C242T with Helicobacter pylori Infection in Esophageal Squamous Cell Carcinoma

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Abstract

Objective: To investigate the interaction of polymorphisms of PPAR- 2 gene -C34G and NADPH oxidase subunit p22phox gene -C242T with Helicobacter pylori (H. pylori) infection in esophageal squamous cell carcinoma (ESCC).

Methods: A total of 200 ESCC patients of each Broder grade (I, II, and III) were enrolled with 200 healthy individuals as controls. Genetic polymorphisms of PPAR- 2 gene -C34G and NADPH oxidase subunit p22phox gene -C242T were analyzed in peripheral blood leukocytes using PCR-RFLP. H. pylori infection status was evaluated by ¹ C-urea breath test (¹ C-UBT) measuring disintegrations per minute (DPM) of ¹ C. Unconditional logistic regression was used to analyze the interaction between nucleotide polymorphisms and H. pylori infection.

Results: The risk of ESCC increased significantly in subjects with -C34G (CG), -C34G (GG), -C242T (CT), and -C242T (TT) genotypes. Combined analysis revealed that subjects carrying -C34G (GG)/-C242T (TT) had a high risk of ESCC, with a positive interaction between -C34G (GG) and -C242T (TT) in increasing ESCC risk. Positive interactions in ESCC pathogenesis were also found between -C34G (CG) and -C242T (TT), between -C34G (CG) and -C242T (CT), and between -C34G (GG) and -C242T (CT) (>1). The risk of ESCC increased significantly in H. pylori-infected subjects, which showed positive interactions with -C34G (CG), -C34G (GG), -C242T (CT), and -C242T (TT) genotypes in increasing ESCC risk (>1).

Conclusion: Individuals carrying -C34G (CG), -C34G (GG), -C242T (CT), and -C242T (TT) genotypes constitute a high-risk population for ESCC. These

genotypes interact with H. pylori infection in ESCC pathogenesis, suggesting the importance of eradicating H. pylori for ESCC prevention.

Keywords: esophageal squamous carcinoma; Helicobacter pylori infection; PPAR- 2 gene -C34G; NADPH oxidase subunit p22phox gene -C242T; gene polymorphisms

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignant tumors in humans, accounting for 2% of all malignant tumors worldwide. Approximately 310,400 new ESCC cases occur annually worldwide, with 167,200 cases in China alone [1]. China is both a high-incidence country for ESCC and has the highest mortality rate from this disease. The etiology and pathogenesis of ESCC remain incompletely understood, though insulin resistance, reactive oxygen species, and lipid peroxidation damage are considered important pathogenic factors closely related to environmental and genetic factors [2-3].

Helicobacter pylori, one of the most common infectious bacteria in the digestive system, has established pathogenic roles in chronic active gastritis, peptic ulcers, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer [4-5]. Recent studies have also found that H. pylori infection is associated not only with gastrointestinal diseases but also with extra-gastrointestinal conditions. H. pylori may participate in the development of malignancies in other sites by affecting lipid metabolism, insulin resistance, inflammatory responses, and oxidative stress [4].

Peroxisome proliferator-activated receptors (PPARs) are a superfamily of ligand-activated transcription factors belonging to the nuclear hormone receptor family. PPAR- 2, predominantly expressed in adipose tissue, is essential for maintaining insulin sensitivity and glucose homeostasis in humans. Mutations or inhibition of the PPAR- 2 gene can lead to insulin resistance and promote the progression of malignant tumors such as ESCC [7]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a key enzyme for reactive oxygen species production in the body, and its activity directly affects ROS levels, playing a major role in tumor development [8].

Both PPAR- 2 and NADPH oxidase genes exhibit polymorphism, possessing multiple alleles that encode proteins with different activities. These polymorphisms may alter individual responses to environmental factors such as H. pylori infection, representing an important determinant of ESCC susceptibility. Although studies on the association between PPAR- 2 and NADPH oxidase gene polymorphisms and ESCC susceptibility are increasing, no reports have examined the combined effects of H. pylori infection and these gene polymorphisms on ESCC susceptibility. To investigate the distribution of PPAR- 2 and NADPH oxidase in the local population and explore their relationship with H.

pylori infection in ESCC development, we conducted the first study in China on the association between combined polymorphisms of these genes and ESCC susceptibility.

Methods

1.1 Diagnostic Criteria

According to Broder' s grading system, ESCC was classified into three grades [9]. **Grade I** squamous carcinoma contains less than 25% atypical squamous cells. The edges of cancer cell nests show intact basal cell arrangement in some areas but disordered arrangement or absence of basal cells in others. Cancer cells are not clearly demarcated from surrounding stroma, with irregular cell arrangement, variable size, and numerous keratin pearls—some completely keratinized, others partially keratinized.

Grade II squamous carcinoma shows cancer cell nests with indistinct boundaries from surrounding stroma, containing 25-50% atypical squamous cells. Keratinization is less prominent with only a few keratin pearls showing incomplete keratinization. The surrounding inflammatory reaction is more pronounced than in Grade I.

Grade III squamous carcinoma contains a large number of atypical squamous cells (50-70%) with minimal or absent keratinization. Keratin pearls are absent, though occasional dyskeratotic cells are visible. Nuclei are atypical with prominent mitotic figures, and the surrounding inflammatory reaction is less marked, indicating diminished host response to cancer cells.

Grade IV squamous carcinoma consists almost entirely of atypical squamous cells without intercellular bridges. Mitotic figures are numerous with complete absence of keratinization. When cancer cells are spindle-shaped, they often show a whorled arrangement.

1.2 Inclusion Criteria

(1) Pathologically confirmed ESCC; (2) Age 39-71 years, both genders; (3) First visit at enrollment without prior chemotherapy or radiotherapy; (4) Signed informed consent.

1.3 Exclusion Criteria

(1) Coexisting benign or malignant tumors or precancerous lesions in other systems; (2) Autoimmune diseases; (3) History of anti-H. pylori medication; (4) Major diseases such as cardiac, renal, or respiratory insufficiency; (5) Local or systemic severe infection; (6) Pregnancy or lactation; (7) Psychiatric disorders or severe neurosis, or inability to communicate due to intellectual or language barriers.

1.4 General Data

According to the above diagnostic, inclusion, and exclusion criteria, ESCC cases treated at the First Affiliated Hospital of Xinxiang Medical University between May 2010 and March 2015 were collected along with healthy controls from the same period. Groups were matched 1:1 by age (±3 years), gender, and residence. The study included 200 cases each of Broder Grade I, Grade II, and Grade III ESCC, plus 200 healthy controls, all residents of northern Henan or having lived there for 10 years. The male-to-female ratio was 127:73 in all groups. Mean ages were: Grade I 49.42±9.83 years (range 41-68), Grade II 49.25±11.46 years (range 40-69), Grade III 48.74±12.26 years (range 42-68), and controls 48.94±12.68 years (range 39-68). No significant differences were found in age, gender, ethnicity, or birthplace among the four groups ($P>0.05$), and subjects were unrelated.

A unified epidemiological questionnaire was administered by trained interviewers to collect data on general characteristics, health-related behaviors, smoking, alcohol consumption, dietary habits, physical activity, and medical history. Dietary factors included: dietary patterns, carbohydrates, vegetables, fruits, meat, eggs, fats, poultry offal, livestock offal, and fried foods. Dietary surveys used interview methods with detailed recording and conversion to food component intake and caloric production. Dietary status was classified as low-fat or high-fat diet (defined as fat-derived calories >25% of total calories for >1 year). Smoking status was categorized as non-smoker or smoker (defined as 1 cigarette daily for >6 months). Alcohol consumption was defined as >140 g ethanol/week for men or >70 g/week for women for >6 months.

1.5 H. pylori Detection

The ^{1} C-UBT kit was provided by Shenzhen Zhonghe Headway Bio-Sci & Tech Co., Ltd. All reagents were prepared according to kit instructions. The detection instrument was a HUBT-01 card-style H. pylori detector (Shenzhen Zhonghe Headway Bio-Sci & Tech Co., Ltd.). Patients were tested after overnight fasting or at least 6 hours of fasting. After rinsing the mouth, subjects swallowed a ^{1} C-urea capsule with 20 mL of water without chewing, waited 3 minutes, drank another 20 mL of water, then sat quietly for 15-20 minutes before sampling.

A CO absorbent bottle was opened and poured into a clean liquid scintillation vial. Subjects exhaled slowly through a catheter into the CO absorbent until the purple-red color disappeared, becoming colorless and transparent, indicating adequate CO collection. Then 4.5 mL of dilute scintillation fluid was added, mixed well, and DPM (disintegrations per minute) was measured. Using the kit' s cutoff of 100 DPM as positive, H. pylori infection status was classified as: non-infected (DPM<100), lightly infected (100 DPM<500), and heavily infected (DPM 500).

1.6 Sample Collection

Venous blood (2-3 mL) was collected from each subject into EDTA-Na anti-coagulant tubes. The leukocyte layer was separated, and leukocyte DNA was extracted using the QIAamp DNA extraction kit. DNA was stored at low temperature for later use.

1.7 Genetic Testing

(1) PPAR- 2 gene -C34G polymorphism analysis [10]: Primer sequences were: forward 5' -CAAGCCCAGTCCTTTCTGTC-3' , reverse 5' -AGTGAAGGAATCGCTTTCCG-3' , synthesized by Research Genetics (USA). The PCR reaction system included: genomic DNA 1 L, each primer 2 L, Taq DNA polymerase Mix (Takara) 6 L. Reaction conditions: initial denaturation at 94°C for 5 min; 40 cycles of 94°C for 45 s, 56°C for 45 s, 72°C for 30 s; final extension at 72°C for 7 min. PCR products were confirmed by Bio-Rad stained agarose gel electrophoresis, then digested with restriction enzyme Hpa at 37°C for 4 h. Digested products (4 L) were analyzed by Bio-Rad stained agarose gel electrophoresis and visualized under UV gel imaging.

(2) NADPH oxidase p22phox subunit gene -C242T polymorphism analysis [11]: Primer sequences were: forward 5'-TGCTTGTGGGTAAACCAAGGCCGGTG-3', reverse 5'-AACACTGAGGTAAGTGGGGGTGGCTCCTGT-3', synthesized by Shanghai Sangon Biotech. PCR amplification was performed using the TaKaRa PCR Amplification Kit (25 L system): 0.5 g genomic DNA, 10×PCR Buffer 2.5 L, MgCl 1.5 L (25 mmol/L), 2 mM dNTP Mixture 2 L, TaKaRa Taq DNA polymerase 0.125 L (5 U/ L), each primer 1 L (10 pmol/ L), sterile deionized water 17.375 L. PCR conditions: 94°C for 4 min; 33 cycles of 94°C for 30 s, 65°C for 1 min, 70°C for 3 min; final extension at 70°C for 10 min. PCR products (12 L) were digested overnight with restriction enzyme Rsa (NEB) at 37°C. Digested products were analyzed by 3.5% agarose gel electrophoresis (containing 0.5 g/mL ethidium bromide).

1.8 Statistical Methods

Hardy-Weinberg equilibrium test was used to assess population representativeness (P>0.05 indicated conformity). Odds ratios (OR) and 95% confidence intervals (95% CI) evaluated relative risk. Genotype and allele frequencies between case and control groups were compared using ^{2} test (P<0.05 indicated statistical significance). Unconditional logistic regression analyzed interactions. The interaction model and coefficient (= eg/ e) proposed by Khoury and Wagener determined gene-environment interaction type [12]: >1 indicated positive interaction (gene amplifies environmental effect); <1 indicated negative interaction; =1 indicated no interaction. In case-control studies, is the ratio of lgOR values. Interaction models were classified as: multiplicative (OReg=ORe×ORg), super-multiplicative (OReg>ORe×ORg), or sub-multiplicative (OReg<ORe×ORg).

Results

2.1 Detection of PPAR- 2 Gene -C34G and NADPH Oxidase p22phox Subunit Gene -C242T Polymorphisms

PPAR- 2 gene -C34G PCR amplification yielded a 267 bp fragment. After Hpa digestion, three genotypes were identified: -C34G (CC) homozygotes showed 224 and 43 bp bands; -C34G (GG) homozygotes showed a single 267 bp band; -C34G (CG) heterozygotes showed 267, 224, and 43 bp bands [Figure 1: see original paper]. Twenty randomly selected samples from each group were verified by DNA sequencing (Shanghai Sangon Biotech) with 100% concordance.

NADPH oxidase p22phox subunit gene -C242T PCR amplification yielded a 348 bp fragment. After Rsa digestion, three genotypes were identified: -C242T (CC) homozygotes showed a single 348 bp band; -C242T (TT) homozygotes showed 188 and 160 bp bands; -C242T (CT) heterozygotes showed 348, 188, and 160 bp bands [Figure 2: see original paper]. Twenty samples per group were verified by sequencing with 100% concordance.

2.2 Analysis of General Data

No significant differences in gender or age distribution were observed between ESCC groups and controls (P>0.05). However, rates of high-fat diet, smoking, and alcohol consumption were significantly higher in Broder Grade I, II, and III groups compared to controls (P<0.05,).

2.3 Correlation Between ESCC Susceptibility and H. pylori Infection

H. pylori infection frequencies (both 100 DPM<500 and DPM 500) were significantly higher in all ESCC grades compared to controls (P<0.01). The risk of ESCC increased significantly in H. pylori-infected subjects (OR=2.0636 for DPM<500; OR=3.1393 for DPM 500 in Grade I; OR=4.4474 and OR=8.0225 in Grade II; OR=10.9677 and OR=21.4583 in Grade III). DPM 500 H. pylori infection conferred significantly higher ESCC risk than 100 DPM<500 infection (P<0.01,).

2.4 Association Analysis of Genotypes and Allele Frequencies

Hardy-Weinberg equilibrium test confirmed that PPAR- 2 gene -C34G genotype distribution in controls conformed to equilibrium (P>0.05), indicating representative population sampling . Significant differences in CC, CG, and GG genotype frequencies existed between case and control groups (P<0.01). Allele G distribution differed significantly between ESCC and control groups (P<0.01) with OR>1, indicating higher ESCC risk in allele G carriers. NADPH oxidase p22phox subunit gene -C242T genotypes and allele frequencies showed similar patterns .

2.5 Multivariate Logistic Regression Analysis

To further analyze ESCC risk factors, H. pylori infection status, -C34G genotype, and -C242T genotype were entered as independent variables with ESCC occurrence as the dependent variable (0: no ESCC; 1: ESCC). Stepwise backward unconditional logistic regression revealed significant associations for H. pylori infection, -C34G, and -C242T [TABLE:5-6].

2.6 Interaction of Gene Polymorphisms in ESCC Pathogenesis

Combined mutation analysis revealed that -C34G (GG)/-C242T (TT) genotype frequencies were 7.00%, 13.50%, 21.00%, and 2.00% in Grades I, II, III, and controls, respectively (all P<0.01). This genotype significantly increased ESCC risk (OR=41.2941 in Grade I; OR=363.9724 in Grade III). A positive interaction existed between -C34G (GG) and -C242T (TT) in ESCC development (^{2}= / , = / ; ^{2}=2.0773 in Grade I; ^{2}=2.0543 in Grade II; ^{2}=2.0004 in Grade III). Additional positive interactions were observed between -C34G (CG) and -C242T (TT), between -C34G (CG) and -C242T (CT), and between -C34G (GG) and -C242T (CT) (all >1) .

2.7 Interaction of H. pylori Infection and Gene Polymorphisms

For H. pylori infection with DPM<500, ORe was 4.7273 in Grade I, 2.2189 in Grade II, and 12.6061 in Grade III. For -C34G (CG) genotype alone, ORg was 2.5267 in Grade I, 6.1176 in Grade II, and 19.8095 in Grade III. When both factors coexisted, interaction OReg was 30.5882 in Grade I, 251.3332 in Grade II, and 5.8367 in Grade III, with interaction coefficient = eg / e>1, indicating a super-multiplicative model (OReg >ORe×ORg). Positive interactions were also observed between 100 DPM<500 H. pylori infection and -C34G (GG), between DPM 500 and -C34G (GG), between 100 DPM<500 and -C34G (CG), between DPM 500 and -C34G (CG), between 100 DPM<500 and -C242T (CT), between DPM 500 and -C242T (CT), and between DPM 500 and -C242T (TT) (all >1) [TABLE:8-9].

Discussion

Insulin resistance may contribute to malignant transformation through hyperinsulinemia, insulin-like growth factor-1, and the IKK- /NF- B signaling pathway [13]. PPAR- 2, a nuclear receptor family member and ligand-induced transcription factor, acts as a fatty acid sensor that regulates target gene transcription, affecting lipid metabolism enzyme and adipocytokine expression. By modulating its activity, PPAR- 2 promotes adipocyte differentiation, prevents lipid accumulation, increases fatty acid oxidation, and improves insulin sensitivity. Additionally, PPAR- possesses anti-proliferative, pro-apoptotic, and pro-differentiation functions, conferring comprehensive anti-cancer activity [14-15].

PPAR- 2 expression is controlled by genetic and environmental factors, with significant individual variation. The human PPAR- 2 gene at 3p25 contains 3 exons and 2 introns. The most common polymorphism is C→G substitution at exon B position 34, causing Pro12Ala substitution. This functional variant affects PPAR- 2 activity, reducing blood and tissue expression and biological effects. The -C34G polymorphism has three genotypes: -C34G (CC), -C34G (CG), and -C34G (GG). Studies have linked -C34G polymorphism to metabolic syndrome [16-17]. Our study found -C34G (CG) and -C34G (GG) associated with ESCC development, consistent with increased metabolic syndrome risk. The mechanism may involve altered PPAR- 2 structure affecting transcriptional activity and insulin sensitivity, as Pro12Ala lies in a ligand-independent, insulin-dependent activation domain where proline prevents -helix formation while alanine promotes it [18].

Excessive reactive oxygen species (ROS) induce nDNA strand breaks, cross-linking, or base modifications, mediating proto-oncogene activation or tumor suppressor inactivation, leading to abnormal cell proliferation [19-20]. ROS also attack membrane phospholipids, initiating lipid peroxidation that damages membranes and causes metabolic dysregulation [20]. NADPH oxidase is the primary ROS source, with the p22phox subunit as its core catalytic component. The C→T mutation at codon 242 on chromosome 16 causes His72Tyr substitution at the heme-binding site, reducing p22phox activity [21]. The -C242T polymorphism increases risk of oxidative stress-related diseases like coronary and cerebrovascular disease [22-23]. Our study found -C242T (CT) and -C242T (TT) associated with ESCC risk (P<0.01), consistent with previous research.

We found that combined -C34G (GG) and -C242T (TT) mutations synergistically increased ESCC risk, with significant mutual amplification effects. Positive interactions also existed between heterozygotes and between heterozygotes and mutant homozygotes (all >1). H. pylori infection alone increased ESCC risk, with DPM 500 infection conferring higher risk than 100 DPM<500 infection (P<0.01). H. pylori infection showed positive interactions with -C34G (CG), -C34G (GG), -C242T (CT), and -C242T (TT) (all >1), with greater hazardous effects in mutation carriers. All interactions followed super-multiplicative models (OReg>ORe×ORg).

H. pylori may cause insulin resistance through chronic inflammation, fibrogenic autoimmune reactions damaging pancreatic cells, and altered gastrointestinal hormones (increased gastrin and decreased somatostatin) that affect insulin secretion [24-25]. H. pylori-induced inflammation recruits neutrophils that produce superoxide radicals, hydrogen peroxide, and hydroxyl radicals, disrupting redox balance and increasing malignant transformation risk [26]. This may explain why H. pylori infection alone and synergistically with PPAR- 2 -C34G and NADPH oxidase -C242T mutations increases ESCC risk.

Our data demonstrate that H. pylori infection and -C34G (CG), -C34G (GG), -C242T (CT), and -C242T (TT) genotypes are risk factors for ESCC, with their interactions increasing disease risk. Further analysis of H. pylori infection

frequency and genotype distributions across Broder grades revealed significant differences (P<0.01), suggesting these factors not only increase ESCC risk but also influence disease progression.

ESCC involves complex interactions between environmental factors and multiple genes. Individuals with PPAR- 2 -C34G and NADPH oxidase -C242T mutations constitute a high-risk population requiring attention in ESCC prevention strategies. While we cannot modify susceptibility genotypes, detecting these mutations can predict individual ESCC risk, enabling targeted interventions such as H. pylori eradication or gene expression modulation for effective prevention.

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