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Glypican-4 gene polymorphism (rs1048369) and susceptibility to Epstein-Barr virus-associated and -negative gastric carcinoma

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ABSTRACT

Background/aims: Gastric cancer (GC) is one of the most common malignant tumors in China and single nucleotide polymorphisms (SNPs) have been found to be highly related to GC carcinogenesis. Glypican-4 (GPC4), a member of the heparan sulphate proteoglycan family, plays an important role in the regulation of cell growth and differentiation. However, little is known about polymorphisms of GPC4 gene and their associated susceptibility to GC, especially to Epstein-Barr virus-associated GC (EBVaGC). Here we studied the GPC4 polymorphism (rs1048369) in GC individuals, especially those with EBVaGC, and we explored an association between the GPC4 gene polymorphism (rs1048369) and susceptibility to EBVaGC and Epstein-Barr virus-negative GC (EBVnGC) in a population from Northern China.
Patients and methods: The GPC4 gene polymorphism (rs1048369) was detected in 54 cases of EBVaGC and 73 cases of EBVnGC using polymerase chain reaction (PCR). One hundred and seven peripheral blood samples from healthy individuals were also measured as a control group.
Results: There were significant differences in both the genotype and allelic frequency of GPC4 gene (rs1048369) between the EBVaGC and EBVnGC patients. Meanwhile, the distribution of genotype and allelic frequency of GPC4 (rs1048369) differed between EBVaGC and control groups. Distribution of the GPC4 genotype also revealed differences between EBVnGC and control groups, no significant differences in the allelic frequency of the GPC4 gene (rs1048369) were observed. The frequency of the T allele in EBVaGC group was significantly higher than that in control and EBVnGC groups.
Conclusions: The GPC4 gene polymorphism and the allele of GPC4 are both associated with susceptibility to EBVaGC. The T allele of GPC4 may represent a risk factor for EBVaGC.
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1. Introduction

GC is one of the most common types of malignant tumors in China. It has become a severe public issue worldwide. It is widely accepted that the major etiological risk factor for GC is microbial infection such as *Helicobacter pylori* bacteria, which can lead to GC through a multistep process that develops from gastritis to gastric atrophy, intestinal metaplasia, dysplasia, and finally to carcinoma (Correa, 1988). Other infections are also likely to be related to gastric tumorigenesis, such as Epstein-Barr virus (EBV), an essential DNA tumor virus and a member of the subfamily of γ-herpes viruses (Epstein et al., 1964). Epidemiological studies have shown that, although half of the world's population suffers from *H. pylori* infection and 95% of the adults are infected with EBV infection, only a small proportion of them progress to chronic atrophic gastritis and ultimately GC (1–2% *H. pylori*-infected cases develop GC and EBVaGC only accounts for 1.3–20.1% in GC) (Scholte et al., 2002; Akiba et al., 2008; Lee et al., 2009; Uozaki and Fukayama, 2008). These data suggest that GC is a multifactorial disease, and that host genetic factors, in response to chronic microbial infection might be involved in the pathogenesis of the tumor; furthermore, genetic factors may play key important roles in GC development.

Recently, many studies have found that gene polymorphisms, especially SNPs, cause the most variation in the human genome;

Abbreviations: GC, gastric cancer; SNPs, single nucleotide polymorphisms; GPC, glypican; EBVaGC, Epstein-Barr virus-associated GC; EBVnGC, Epstein-Barr virus-negative GC; HSPGs, heparan sulphate proteoglycans; GRIPS, glypican-related integral membrane proteoglycan family; SGBS, Simpson–Golabi–Behmel syndrome; OR, odds ratio; HSPG, heparan sulphate proteoglycan; HS-GAG, heparan sulphate glycosaminoglycan; FGFs, fibroblast growth factors; WNTs, wingless-types; BMPs, bone morphogenetic proteins.

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thus, they have been studied as the key causative factor at the early stage of GC carcinogenesis (Wang et al., 2013; Wu et al., 2013; Zhou et al., 2012; Mu and Su, 2012). SNPs have been widely used as biomarkers for diagnosis and to evaluate the risk and prognosis of some diseases and cancers (Xu et al., 2015; Pipan et al., 2015). Glypicans (GPCs) are cell-bound heparan sulphate proteoglycans (HSPGs) that are evolutionarily conserved in organisms such as nematodes, fruit flies, and mammals (Hacker et al., 2005; Bulow and Hobert, 2006). For vertebrates, there are typically six glypican genes (gpc1 to gpc6) that play an important role in the regulation of cell growth and differentiation, and these genes might be involved in the genesis of cancer. Glypican-5 (GPC5) polymorphisms have been found to be associated with lung cancer (Li and Yang, 2011). The loss of glypican-3 (GPC3) is related to some malignant tumors, such as mesothelioma, ovary, and breast cancers (Filmus, 2001). Indeed, the overexpression of GPC3 acts as an oncogene in hepatocellular carcinomas where it is regarded as a new diagnostic molecular marker (Jakubovic and Jothy, 2007; Jia et al., 2007). Although it is another important member of the heparan sulphate proteoglycan family, the function of GPC4 is rarely explored. Cell surface HSPGs are composed of a membrane-associated protein core that is substituted with a variable number of heparan sulphate chains. Members of the glypican-related integral membrane proteoglycan family (GRIPS) contain a core protein anchored to the cytoplasmic membrane via a glycosyl phosphatidylinositol linkage. These proteins may play a role in the regulation of cell division and growth. GPC4 gene is adjacent to the 3′ end of GPC3 and may also play a role in Simpson–Golabi–Behmel syndrome (SGBS) (Gene ID: 2239). The role of GPC4 in GC genesis has not yet been explored. The purpose of the present study was to evaluate the GPC4 gene polymorphism (rs1048369) in GC individuals in Northern China, especially in EBVaGC cases. Furthermore, an association between the GPC4 polymorphism (rs1048369) and susceptibility to EBVaGC and EBVnGC was also explored.

2. Materials and methods

2.1. GC tissue samples

One hundred and twenty-seven gastric tumor tissues were obtained from the Affiliated Hospital of Qingdao University from January 2002 to March 2014. 76 males and 51 females were included. The mean age was 50.07 ± 18.67 years (range, 26–86 years). None of the patients received hormone therapy, chemotherapy, or radiotherapy before surgery. Peripheral blood samples from 107 healthy individuals, including 60 males and 47 females with no history of gastric disease, were classified as a control group, and this group did not receive any medications. The mean age of the control group was 51.45 ± 11.64 years (range, 30–83 years). This study was approved by the Medical Ethical Committee of Medical College, Qingdao University. The GC and healthy peripheral blood samples were collected after written informed consent was obtained from all subjects. Histological diagnosis and pathological stages of all collected tissues were determined by at least two independent pathologists. Using in situ hybridization of EBV-encoded small RNA 1, as previously described (Parkin et al., 2005), 54 gastric cancer tissue samples were identified as EBVaGC and 73 cases were identified as EBVnGC.

2.2. DNA extraction

DNA was extracted from fresh tumor tissues and whole blood specimens by a standard method using proteinase K digestion and phenol-chloroform purification. A QIAamp DNA FFPE Tissue Kit (QIAGEN GmbH, Hilden, Germany) was used to extract the DNA from paraffin-embedded tumor tissues. The extracted DNA was then prepared for next PCR.

2.3. PCR amplification

The PCR technique was used to detect the GPC4 gene polymorphism [11869C/T (rs1048369)]. PCR was performed with 1.5 μL of DNA extract (100 ng/μL) in a 25 μL reaction mixture containing a standard PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM of each primer, and 1.0 U Taq DNA polymerase. The DNA amplification protocol included 1 cycle at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min. The program ended with a final extension at 72 °C for 10 min. Specific primers for PCR were obtained from Jinsirui (Nanjing, China). Primer sequences were as follows: GPC4, forward 5′-ATCTTCACGCCTGTAATCC-3′, reverse 5′-CCACTTCCACTTCCTTCTC-3′. PCR products were analyzed via electrophoresis on a 2% agarose gel. After electrophoresis, the gels were stained with ethidiumbromide and photographed under a UV light transilluminator. Sterile double distilled water was used as a negative control in each PCR. PCR products were analyzed on an ABI 3730 DNA sequencer to confirm genotype identity.

2.4. Statistical analysis

The Chi-square test was used to compare differences between each group regarding genotype and allelic frequencies. Unconditional logistic regression was used to compare the odds ratio (OR) and P values to indicate the correlation between gene polymorphism and risk of GC. Significance was set at $P < 0.05$. Statistical analysis was conducted using SPSS 18.0 statistical software (SPSS, Chicago, IL, USA).

3. Results

3.1. Confirmation of GPC4 PCR products

The expected size of the amplification product of the GPC4 gene polymorphism (rs1048369) was 400 bp (see Supplementary Fig. S1 in the online version at Doi: 10.1016/j.virusres.2016.04.005). All 54 cases of EBVaGC, 73 cases of EBVnGC, and 107 cases of blood donors were positive for this band.

3.2. Genotypic and allelic distribution of GPC4 (rs1048369)

Determination of the GPC4 genotype was based on the sequencing analysis results using DNA STAR software. The representative sequences are shown in Fig. 1. To explore the relationship between EBV infection and GPC4 gene polymorphism (rs1048369) in GC development, we analyzed differences in the GPC4 genotype (rs1048369) distribution between EBVaGC and EBVnGC cases. The significantly different distribution of GPC4 genotypes between EBVaGC and EBVnGC cases is presented in Table 1 ($\chi^2 = 11.03$, $P = 0.004$). The percentage of the CC, CT, and TT genotype in 54 EBVaGC cases was 48.1%, 0%, and 51.9%, respectively, while the corresponding proportion was 60.3%, 11.0%, and 28.7% in 73 EBVnGC cases. Individuals with the TT genotype showed a significant association with a 2.26-fold increased risk of EBVaGC (P = 0.03; OR = 2.26; 95% CI = 1.07–4.75). Furthermore, the CT genotype showed a decreased risk of EBVaGC (P = 0.04; OR = 0.69; 95% CI = 0.53–0.75). These results indicate that the rs1048369 polymorphism of GPC4 gene is highly associated with EBVaGC in the North China population. Further statistical analysis on the GPC4 (rs1048369) genotype distribution among EBVaGC, EBVnGC and control groups showed that obvious differences could be observed between both EBVaGC and controls ($\chi^2 = 30.07$, $P = 0.000$) as well as

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