

Detection of a 4-bp Insertion/deletion Polymorphism within the Promoter of EGLN2 Using Mismatch PCR-RFLP and Its Association with Susceptibility to Breast Cancer

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Abstract

It has been shown that a 4-bp insertion/deletion (ins/del) polymorphism of EGLN2 influences the risk of several cancers. However, to date, no study has inspected the impact of the 4-bp ins/del polymorphism on breast cancer (BC) risk. A case-control study, including 134 breast cancer patients and 154 healthy women, was here conducted to examine the possible association between EGLN2 4-bp ins/del polymorphism and BC risk in a southeast Iranian population. A mismatched polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was designed for genotyping of the variant. Our findings did not support any association between the 4-bp ins/del polymorphism and the risk of BC in the codominant, dominant, recessive and allele inheritance models tested. When links between the EGLN2 4-bp ins/del polymorphism and clinicopathological characteristics of the patients were evaluated the variant was only associated with HER2 status. More studies with larger sample sizes and diverse ethnicities are warranted to verify our finding.

Keywords: EGLN2- breast cancer- polymorphism- deletion

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Introduction

Breast cancer (BC), the most common cancer in women, is identified as the second cause of cancer mortality among women worldwide (Bray et al., 2013). BC is the commonest cancer among Iranian female involving 21.4% of all cancers (Babu et al., 2011). Though the precise etiology of BC is unrevealed, it has been suggested that genetic factors play critical role in the development and progression of BC (Omrani et al., 2014; Eskandari-Nasab et al., 2015; Rezaei et al., 2016).

Hypoxia is a main feature of solid tumors which induces alterations of gene expression in tumor cells to acclimate to the hypoxic environment (Brahimi-Horn et al., 2007). The hypoxia-inducible factor 1 (HIF-1) is a major transcriptional activator of genes that are induced by hypoxia (Semenza, 1999). The HIF-1 playing a key roles in the development of solid tumors and coordinating the cellular response to hypoxia and oxygen homeostasis (Maxwell and Ratcliffe, 2002; Semenza, 2007; Kaelin and Ratcliffe, 2008). The expression level of HIF-1 is regulated firmly by three prolyl-hydroxylase domain enzymes (PHDs), PHD1, PHD2 and PHD3 (Appelhoff et al., 2004; Willam et al., 2004). Prolyl hydroxylases (PHDs) are involved in the catalyze degradation of HIF-1 by prolyl

hydroxylation of specific residues (Appelhoff et al., 2004; Stolze et al., 2006).

PHD1 is encoded by EGLN2 (Egl nine homolog 2) gene which is mapped to chromosome 19q13.2 (Ryan et al., 2014). A 4-bp ins/del polymorphism (rs10680577) of EGLN2 have been revealed to be associated with the risk of cancers including hepatocellular carcinoma (HCC) (Zhu et al., 2012), non-small cell lung cancer (Che et al., 2014) and colorectal cancer (Li et al., 2017). To the best of our knowledge, there is no report regarding the impact of rs10680577 variant on BC risk. Therefore, we conducted a case-control study to investigate the possible associations between the rs10680577 polymorphism and BC risk in a sample of southeast Iranian population.

Materials and Methods

This case-control study conducted on 134 histologically confirmed BC patients and 154 ages matched healthy women. The enrollment process and study design have been previously reported elsewhere (Sanaei et al., 2016; Hashemi et al., 2017; Sanaei et al., 2017). Ethical approvals for recruitment were taken from local Ethics Committee of Zahedan University of Medical Sciences, and informed consent was obtained from all participants.

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Blood samples were gathered in EDTA tube, and genomic DNA was extracted by salting out method.

Genotyping

We designed mismatch polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for genotyping of rs10680577 (4-bp ins/del) polymorphism within the promoter of EGLN2 gene. Mismatched C was introduced into the forward primers at -4 bp from the polymorphic site to create *AleI* restriction site. The forward and reverse primers were 5'-CCGTTATAAAAGATACTTATGTAAATCAC-3' and 5'-TTGGAATCAAGTGGCGTCG-3', respectively.

Each 0.20 ml PCR reaction tube consisted of 1 µl of genomic DNA (~100 ng/ml), 1 µl of each primer (10 µM), 10 µl of 2X Prime Taq Premix (Genet Bio, Korea) and 7 µl ddH₂O. The PCR conditions were 95 °C for 5 min, followed by 30 cycle of 30 s at 95°C, 30s at 57°C, and 30s at 72 °C, with a final extension step at 72 °C for 5 min. The PCR product (10 µl) was digested by *AleI* restriction enzyme (New England BioLabs, Beverly, MA). The digested products were electrophoresed on 2.5% agarose gel containing 0.5 µg/mL ethidium bromide, visualized on a UV transilluminator and photograph was taken Figure 1. The del allele digested and produced 224 and 31 bp fragments while the ins allele undigested (259 bp). For the quality control of genotyping, approximately 20% of the random samples were re-genotyped and the reproducibility was 100%.

Statistical analysis

The SPSS 22 statistical package was used to achieve statistical analyses. Independent sample t-test and the χ^2 test were used for continuous and categorical data, respectively. Allele and genotype frequency distributions of the variants in patients and controls were determined by χ^2 tests and expressed as percentages of the total number of alleles and genotypes. Odds ratios (ORs) and 95% confidence intervals (95% CIs) was calculated by unconditional logistic regression analysis. P value less than 0.05 was considered to be statistically significant.

Results

The study group consisted of 134 BC patients with an average age of 49.0 ±11.2 years and 154 healthy women with a mean age of 47.4 ±11.6 years. No statistically significant difference was found between the groups

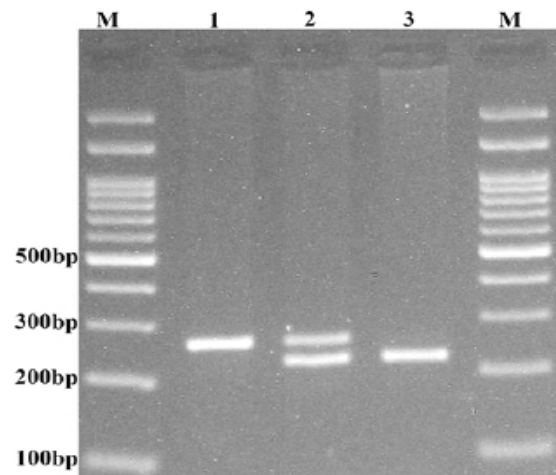


Figure 1. Photograph of the EGLN2 rs10680577 (4-bp ins/del) Polymorphism Using Mismatch Polymerase Chain Reaction-restriction Fragment Length Polymorphism (PCR-RFLP). M: DNA marker; lane1: ins/ins genotype; lanes 2: ins/del; lanes 3: del/del.

regarding age (p=0.218), which displays satisfactory frequency matching.

The genotype and allelic frequency distribution of ins/del polymorphism of EGLN2 in the cases and the controls are shown in Table 1. We did not observe significant differences in the genotype and allele frequencies between BC patients and controls (P>0.05).

The association between the EGLN2 4-bp ins/del polymorphism and clinicopathological characteristic including age, TNM stage, tumor grade, estrogen and progesterone receptors status as well human growth factor receptor 2 (HER2) status were determined. The findings showed only a significant association between EGLN2 4-bp ins/del polymorphism and HER2 status Table 2.

Discussion

To our knowledge, this is the first study investigating the association of EGLN2 4-bp ins/del polymorphism with the risk of BC. Our findings did not support an association between EGLN2 polymorphism and the risk of BC. Zhu et al., (2012) observed that the 4-bp del allele was significantly associated with the risk of hepatocellular carcinoma (HCC) and PHD1 expression.

Li et al., (2017) have found that ins/del as well as del/del genotype significantly increased the risk of

Table 1. Genotype and Allele Frequencies of EGLN2 rs10680577 (4-bp ins/del) Polymorphism in BC and Controls

rs10680577 polymorphism	Case n (%)	Control n (%)	OR (95%CI)	P-value
ins/ins	35 (26.1)	50 (32.5)	1.00	-
ins/del	94 (70.1)	91 (59.1)	1.48 (0.88-2.48)	0.151
del/del	5 (3.7)	13 (8.4)	0.55 (0.18-1.68)	0.425
ins/del+del/del	99 (73.8)	104 (67.5)	1.36 (0.81-2.27)	0.247
Allele				
ins	164 (61.2)	191 (62.0)	1.00	-
del	104 (38.8)	117 (38.0)	1.04 (0.74-1.45)	0.863

OR, odds ratio; ins, insertion; del, deletion.

Table 2. Correlation between EGLN2 rs10680577 (4-bp ins/del) Polymorphism and Clinical Characteristics of Breast Cancer Patients

Characteristics of patients	EGLN2 rs10680577			p-value
	Ins/ins	Ins/del	del/del	
Age, years				0.487
≤ 50	31	41	1	
>50	21	51	4	
Tumor Size (cm)				0.475
≤ 2	9	29	1	
>2	23	47	4	
Grade				
I	8	8	1	0.691
II	19	48	2	
III+IV	5	14	1	
Stage				
I	4	15	1	0.925
II	17	40	1	
III	8	24	2	
IV	6	14	1	
Histology				0.700
Ductal carcinoma	26	67	3	
Other	8	24	2	
Estrogen Receptor status				0.432
Positive	19	59	2	
Negative	15	33	3	
Progesterone receptor status				0.152
Positive	14	55	3	
Negative	20	36	2	
HER2 status				0.029
Positive	21	36	4	
Negative	14	57	1	

Ins, insertion; del, deletion

colorectal cancer (CRC). Wang et al., (2014) reported that homozygote 4-bp del/del confer a significantly increased risk of gastric cancer (GC) in the Chinese population. Che et al.,(2014) have found that the heterozygote ins/del genotype is significantly correlated with increased risk of non-small cell lung cancer (NSLC) risk.

Findings have indicated that the expression level of PHD1 is related to tumorigenesis or poor prognosis (Zhang et al., 2009; Gossage et al., 2010; Andersen et al., 2011; Chen et al., 2011; Peurala et al., 2012; Kaufmann et al., 2013). The 4-bp ins/del (rs10680577) is positioned at -1641 bp upstream of the transcription start site of EGLN2 gene. So, the genotype–phenotype relationship could be mediated by a distinction promoter polymorphism-associated regulatory mechanism. As this variant is situated within the intronic area of RERT-lncRNA, it is reasonable that rs10680577 may impact on RERT-lncRNA expression by affecting its folding structures, which in turn affect EGLN2 expression (Zhu et al., 2012).

Expression of PHD1 is also related to high proliferation of BC (Peurala et al., 2012). Zhang et al., (2009) have

shown that PHD1 activity which is estrogen inducible in breast cancer increases cell proliferation via the regulation of cyclin D1. Loss of PHD1 activity reduces Cyclin D1 expression, consequently decreasing the happening of BC (Zhang et al., 2009).

In summary, our findings suggested that EGLN2 4-bp ins/del polymorphism was not correlated with the risk of BC in a sample of southeast Iranian population. Replication in different populations with larger sample sizes are required for understanding the impact of EGLN2 4-bp ins/del polymorphism on BC risk.

Disclosure of Conflicting Interests

The Authors declare that there is no conflict of interest to disclose.

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References

- Andersen S, Donnem T, Stenvold H, et al (2011). Overexpression of the HIF hydroxylases PHD1, PHD2, PHD3 and FIH are individually and collectively unfavorable prognosticators for NSCLC survival. *PLoS One*, **6**, e23847.
- Appelhoff RJ, Tian YM, Raval RR, et al (2004). Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. *J Biol Chem*, **279**, 38458-65.
- Babu GR, Samari G, Cohen SP, et al (2011). Breast cancer screening among females in Iran and recommendations for improved practice: a review. *Asian Pac J Cancer Prev*, **12**, 1647-55.
- Brahimi-Horn MC, Chiche J, Pouyssegur J (2007). Hypoxia and cancer. *J Mol Med (Berl)*, **85**, 1301-7.
- Bray F, Ren JS, Masuyer E, et al (2013). Global estimates of cancer prevalence for 27 sites in the adult population in 2008. *Int J Cancer*, **132**, 1133-45.
- Che J, Jiang D, Zheng Y, et al (2014). Polymorphism in PHD1 gene and risk of non-small cell lung cancer in a Chinese population. *Tumour Biol*, **35**, 8921-5.
- Chen S, Zhang J, Li X, et al (2011). The expression of prolyl hydroxylase domain enzymes are up-regulated and negatively correlated with Bcl-2 in non-small cell lung cancer. *Mol Cell Biochem*, **358**, 257-63.
- Eskandari-Nasab E, Hashemi M, Amininia S, et al (2015). Effect of TP53 16-bp and beta-TrCP 9-bp INS/DEL polymorphisms in relation to risk of breast cancer. *Gene*, **568**, 181-5.
- Gossage L, Zaitoun A, Fareed KR, et al (2010). Expression of key hypoxia sensing prolyl-hydroxylases PHD1, -2 and -3 in pancreaticobiliary cancer. *Histopathology*, **56**, 908-20.
- Hashemi M, Amininia S, Ebrahimi M, et al (2017). Association between polymorphisms in TP53 and MDM2 genes and susceptibility to prostate cancer. *Oncol Lett*, **13**, 2483-9.
- Kaelin WG Jr, Ratcliffe PJ (2008). Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell*, **30**, 393-402.
- Kaufmann MR, Schraml P, Hermanns T, et al (2013). Onconeural antigen Cdr2 correlates with HIF prolyl-4-hydroxylase PHD1 and worse prognosis in renal cell carcinoma. *Exp Mol Pathol*, **94**, 453-7.
- Li C, Feng L, Niu L, et al (2017). An insertion/deletion polymorphism within the promoter of EGLN2 is associated with susceptibility to colorectal cancer. *Int J Biol Markers*, **32**, e274-e7.
- Maxwell PH, Ratcliffe PJ (2002). Oxygen sensors and angiogenesis. *Semin Cell Dev Biol*, **13**, 29-37.
- Omrani M, Hashemi M, Eskandari-Nasab E, et al (2014). hsa-mir-499 rs3746444 gene polymorphism is associated with susceptibility to breast cancer in an Iranian population. *Biomark Med*, **8**, 259-67.
- Peurala E, Koivunen P, Bloigu R, et al (2012). Expressions of individual PHDs associate with good prognostic factors and increased proliferation in breast cancer patients. *Breast Cancer Res Treat*, **133**, 179-88.
- Rezaei M, Hashemi M, Sanaei S, et al (2016). Association between vascular endothelial growth factor gene Polymorphisms with breast cancer risk in an Iranian population. *Breast Cancer (Auckl)*, **10**, 85-91.
- Ryan DM, Vincent TL, Salit J, et al (2014). Smoking dysregulates the human airway basal cell transcriptome at COPD risk locus 19q13.2. *PLoS One*, **9**, e88051.
- Sanaei S, Hashemi M, Eskandari E, et al (2017). KRAS gene polymorphisms and their impact on breast cancer risk in a sample of Iranian population. *Asian Pac J Cancer prev*, **18**, 1301-5.
- Sanaei S, Hashemi M, Rezaei M, et al (2016). Evaluation of the pri-miR-34b/c rs4938723 polymorphism and its association with breast cancer risk. *Biomed Rep*, **5**, 125-9.
- Semenza GL (1999). Perspectives on oxygen sensing. *Cell*, **98**, 281-4.
- Semenza GL (2007). Hypoxia-inducible factor 1 (HIF-1) pathway. *Sci STKE*, **2007**, cm8.
- Stolze IP, Mole DR, Ratcliffe PJ (2006). Regulation of HIF: prolyl hydroxylases. *Novartis Found Symp*, **272**, 15-25.
- Wang J, Zhang J, Zhou C, et al (2014). An insertion/deletion polymorphism within the proximal promoter of EGLN2 is associated with susceptibility for gastric cancer in the Chinese population. *Genet Test Mol Biomarkers*, **18**, 269-73.
- Willam C, Nicholls LG, Ratcliffe PJ, et al (2004). The prolyl hydroxylase enzymes that act as oxygen sensors regulating destruction of hypoxia-inducible factor alpha. *Adv Enzyme Regul*, **44**, 75-92.
- Zhang Q, Gu J, Li L, et al (2009). Control of cyclin D1 and breast tumorigenesis by the EglN2 prolyl hydroxylase. *Cancer Cell*, **16**, 413-24.
- Zhu Z, Gao X, He Y, et al (2012). An insertion/deletion polymorphism within RERT-lncRNA modulates hepatocellular carcinoma risk. *Cancer Res*, **72**, 6163-72.



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