RESEARCH ARTICLE

# No Association of Hypoxia Inducible Factor-1α Gene Polymorphisms with Breast Cancer in North-West Indians

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## Abstract

**Background: Hypoxia inducible factor-1 alpha (*HIF-1α*) is the key regulator of cellular responses to hypoxia and plays a central role in tumour growth. Presence of Single nucleotide polymorphisms (SNPs) in the critical regulatory domains of *HIF-1α* may result in the overexpression of the protein and subsequent changes in the expression of the downstream target genes. The aim of study was to investigate the association of three SNPs (g.C111A, g.C1772T and g.G1790A) of *HIF-1α* with the risk of breast cancer in North Indian sporadic breast cancer patients. Materials and Methods: A total of 400 subjects, including 200 healthy controls and 200 patients with breast cancer were recruited in this study. Genotypes were determined using polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) method. Results: The CC and CA genotype frequency of *HIF-1α* g.C111A polymorphism was 100 *vs* 99% and 0 *vs* 1% in breast cancer patients and healthy controls respectively. The frequencies of CC, CT and TT genotype of g.C1772T polymorphism were 76 *vs* 74.5%, 19 *vs* 21% and 5 *vs* 4.5% in breast cancer patients and control individuals respectively. There was no significant difference in genotype and allele frequencies of *HIF-1α* g.C1772T polymorphism between cases and control individuals (p>0.05). For g.G1790A genotypes, all patients and controls had only GG genotype. Conclusions: The three *HIF-1α* polymorphisms (g.C111A, g.C1772T and g.G1790A) are not associated with breast cancer risk in North-West Indian patients.**

**Keywords:** Breast cancer - hypoxia - polymorphism - lack of relationship

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## Introduction

Intratumoral hypoxia, the pathophysiologic consequence of the structurally and functionally disturbed microcirculation is a hallmark of most of solid tumors (Hill et al., 2009). Hypoxia within the tumor microenvironment plays a critical role in the upregulation of several chemokine receptors on tumor cells and secretion of different chemokines promoting tumor cell invasion and metastasis (Newcomb and Zagzag, 2009). Hypoxia-inducible factor-1 (HIF-1), a regulator of chemokine receptor expression has been reported to up-regulate several genes associated with tumour progression, glycolysis, angiogenesis, and metastasis (Semenza and Wang, 1992; Semenza, 2003; Wenger et al., 2005; Rankin and Giaccia, 2008). HIF-1 is a heterodimeric, helix-loop-helix transcription factor consisting of α and β subunits. The β subunit is constitutively expressed and α subunit which determines HIF-1 activity is regulated by oxygen tension.

*HIF-1α* (OMIM 603348) is mapped to 14q23.2 and consists of 15 exons. *HIF-1α* is hydroxylated and degraded rapidly under normoxic conditions through von Hippel-Lindau (VHL) mediated ubiquitin-proteasome pathway whereas under hypoxic conditions it becomes stabilized and is rapidly accumulated in cell (Tanimoto et al., 2003; Smaldone and Maranchie, 2009). Overexpression of *HIF-1α* has been documented in various cancers probably as a consequence of intratumoral hypoxia or genetic alterations (Zhong et al., 1999; Talks et al., 2000; Poon et al., 2009; Ruan et al., 2009).

Predisposition to several human cancers have been associated with genetic polymorphisms, which may represent an important contributor to cancer susceptibility and tumor behavior (Medeiros et al., 2003, 2004; Pinto et al., 2004; Santos et al., 2006). The association of three (p.S28Y, p.P582S, p.A588T) nonsynonymous polymorphisms of *HIF-1α* with cancer susceptibility and prognosis has been investigated individually but the results are inconsistent. The g.C111A (p.S28Y) lies within the critical region of the basic-helix-loop-helix (bHLH) domain in exon 2 whereas g.C1772T (p.P582S) and g.G1790A (p.A588T) are located within oxygen-dependent degradation domain in exon 12

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of *HIF-1α*. Presence of these variants in the critical regulatory domains may result in the overexpression of the protein and subsequent changes in the expression of the downstream target genes (Chau et al., 2005). Association of g.C1772T and g.G1790A polymorphisms with significantly higher transcriptional activities and enhanced angiogenesis than the wild type under both normoxic and hypoxic conditions has also been reported (Tanimoto et al., 2003; Smaldone and Maranchie, 2009). The enhancement of transactivation capacity associated with g.C1772T and g.G1790A polymorphisms might be due to alteration of protein stability of these variant proteins or due to enhanced recruitment of transcriptional co-factors such as CBP/p300 and SRC-1 that interact with *HIF-1α* by the variant forms via conformational changes caused by amino acid substitution (Carrero et al., 2000). The enhancement in protein stability of these variants may result from the effects of the mutations within this regulatory region interfering with different post-translational modifications of *HIF-1α* (Chau et al., 2005). In prostate cancer, it has been found that patients with TT genotype had significantly higher *HIF-1α* mRNA expression than those with CC genotype (Vainrib et al., 2012). A similar result was also reported for patients with breast cancer (Kim et al., 2008). In a meta-analysis, p.P582S polymorphism was found to be associated with urinary cancer risk in Caucasian population, while p.A588T polymorphism was found to be associated with increased risk of developing urinary cancers in Asians (Li et al., 2013). A recent meta-analysis indicated the potential role of *HIF-1α* g.C1772T polymorphism with increased risk of developing malignancy in Asians (Wu et al., 2014).

In Amritsar district of Punjab, a major agragarian state in North-West India, Population Based Cancer Registry (PBCR) has reported a higher incidence of cancer (81.2 per lakh) (http://www.downtoearth.org.in/content/punjab-cancer-capital-india). The frequency of sporadic breast cancer is also increasing in the Amritsar district of Punjab state (personal communication, SGRD Rotary Cancer Hospital, Vallah, Sri Amritsar). Hypoxia-inducible factor-1 (HIF-1) a regulator of chemokine receptor expression has been reported to up-regulate more than 80 genes associated with tumour progression, glycolysis, angiogenesis, and metastasis (Semenza and Wang, 1992; Semenza, 2003; Wenger et al., 2005; Rankin and Giaccia, 2008). *HIF-1α* plays a major role in chemokine-chemokine receptor systems (Newcomb and Zagzag, 2009). Single nucleotide polymorphisms may be associated not only with inter-individual predisposition to breast cancer, but also with phenotypic traits, treatment outcomes with anticancer agents and disease prognosis. To date, no reported study has examined the combined role of p.S28Y, p.P582S and p.A588T polymorphisms of *HIF-1α* in breast cancer. Since *HIF-1α* plays a critical role in the development and progression of cancer, the present study was aimed to elucidate the association of p.S28Y, p.P582S and p.A588T polymorphisms of *HIF-1α* with the risk to breast cancer in North Indian sporadic breast cancer patients.

## Materials and Methods

*Study subjects*

In this hospital based case control study, patients were selected from Sri Guru Ram Das Institute of Medical Sciences and Research, Vallah, Amritsar, Punjab. Two hundred clinically confirmed sporadic breast cancer patients and 200 age and gender matched normal healthy individuals were recruited as study subjects. Patients who had received chemotherapy, radiotherapy or blood transfusion before surgery or had previous history of any malignancy were excluded from the study. Controls were biologically unrelated to cancer patients and were from same geographical region as that of patients. Individuals with family history of any cancer or other chronic disease were not included in the control group. Epidemiological data was collected from each subject using pre-tested structured questionnaire which included demographic particulars, family history, disease history etc. After informed consent, 5 ml peripheral venous blood sample was collected from each subject in 0.5M EDTA. This study was undertaken after approval by the institutional ethical committee of Guru Nanak Dev University, Amritsar, Punjab, India.

*Genomic DNA extraction and genotyping of HIF-1α polymorphisms*

The genomic DNA was extracted from peripheral blood lymphocytes using standard phenol chloroform method (Adeli and Ogbonna, 1990). Three polymorphisms (g.C111A, g.C1772T and g.G1790A) of *HIF-1α* were screened by PCR-RFLP method using the published primer sequences (Apaydin et al., 2008). A negative control without template DNA was included in each reaction to monitor contamination. To ensure quality control, genotyping was performed without knowledge of case/control status.

*Analysis of g.C111A (p.S28Y) polymorphism*

The PCR reaction was set in 15μl reaction volume containing 50ng DNA, 1X Taq buffer with 1.5 mM $MgCl_2$, 1.2μl dNTPs mixture (Bangalore GeNei), 6 picomole of each primer (Sigma, St. Louis, MO, USA), 1 U Taq DNA Polymerase (Bangalore GeNei). The PCR conditions were initial denaturation at 95°C for 5 min followed by 35 cycles with denaturation at 95°C for 45s, annealing at 59°C for 30 s and extension at 72°C for 45s, and final extension at 72˚C for 10 min in a Mastercycler gradient, (Eppendorf, Germany). The PCR products were analyzed on 2% ethidium bromide stained agarose gel. Amplified products were digested with *Bgl*II restriction enzyme following the manufacturer instructions (New England Biolabs, Beverly, MA). The restriction digestion reaction products were analyzed on 2.3% ethidium bromide stained agarose gel. The presence of the C allele was indicated by bands of 143 and 44 base pairs, whereas undigested product of 187bp indicated the A allele.

*Analysis of g.C1772T (rs11549465) and g.G1790A (rs11549467) polymorphism*

The PCR reaction mixture of 25μl was prepared by

adding 100ng of DNA, 1X Taq buffer with 1.5 mM $MgCl_2$, 2$\mu$l dNTPs mixture (Bangalore GeNei), 10 picomole of each primer (Sigma) and 1.5 U of Taq DNA polymerase (Bangalore GeNei). The PCR conditions were initial denaturation at 95°C for 5 min followed by 35 cycles with denaturation at 95°C for 45s, annealing at 55°C for 30s and extension at 72°C for 45s, and final extension at 72˚C for 10 min in a Mastercycler gradient, (Eppendorf, Germany). For analysis of g.C1772T and g.G1790A polymorphism, amplified products were digested with *Hph*I and *Aci*I restriction enzyme respectively (New England Biolabs, Beverly, MA). The restriction digestion reaction products were analyzed on 2.3% ethidium bromide stained agarose gel. For g.C1772T polymorphism, the presence of the C allele was indicated by bands of 228 and 118 base pairs, whereas undigested product of 346bp indicated the T allele. For g.G1790A polymorphism, the presence of the G allele was indicated by bands of 201 and 145 base pairs, whereas undigested product of 346bp indicated the A allele.

**Table 1. Characteristics of Breast Cancer Patients and Controls**

| Characteristics | Patients n=200 | Controls n=200 | p value |
|---|---|---|---|
| Gender | | | |
| Males | 6 (3) | 6 (3) | 1 |
| Females | 194 (97) | 194 (97) | |
| Age in Years | | | |
| <40 | 42 (21) | 42 (21) | |
| 40-49 | 61 (30.5) | 61 (30.5) | |
| 50-59 | 48 (24) | 48 (24) | |
| 60-69 | 34 (17) | 34 (17) | |
| 70-79 | 14 (7) | 14 (7) | |
| 80-89 | 1 (0.5) | 1 (0.5) | |
| Mean ± SD | 49.05±11.70 | 49.03±11.69 | 0.99 |
| Range | 25-85 | 25-85 | |
| Habitat | | | |
| Rural | 134 (67) | 134 (67) | 1 |
| Urban | 66 (33) | 66 (33) | |
| Diet | | | |
| Vegetarian | 127 (63.5) | 112 (56) | 0.13 |
| Non-Vegetarian | 73 (36.5) | 88 (44) | |
| Menopausal status | | | |
| Premenopausal | 80 (41.2) | 90 (46.39) | 0.31 |
| Postmenopausal | 114 (58.8) | 104 (53.61) | |
| Tumor stage | | | |
| I | 23 (11.5) | | |
| II | 104 (52) | - | |
| III | 54 (27) | | |
| IV | 19 (9.5) | | |
| Histological Type | | | |
| Invasive Ductal carcinoma | 189 (94.5) | - | |
| Invasive Lobular carcinoma | 4 (2.0) | | |
| Others | 7 (3.5) | | |

*Data are presented as number (percentage) or mean±Standard deviation

*Statistical analysis*

The statistical analysis was done to evaluate the association of screened polymorphisms with breast cancer risk. Hardy Weinberg Equilibrium (HWE) was tested by comparing the observed to expected genotype frequencies using the Chi-square ($\chi^2$) test. This test was also used to demonstrate the significant difference of genotype and allele frequencies between the breast cancer patients and normal controls. The odds ratio (OR) with 95% confidence interval (CI) were calculated to determine the association between *HIF-1α* polymorphisms with breast cancer risk. A value of $p \leq 0.05$ was considered statistically significant. All the statistical values were calculated using SPSS Version 16 (SPSS Inc, Chicago, IL).

## Results

*Characteristics of study subjects*

Our study group consisted of 200 patients with pathologically confirmed breast cancer and 200 healthy control subjects. The characteristics of breast cancer patients and controls are summarized in Table 1. Of 200 breast cancer patients 194 (97%) were females whereas 6 (3%) were males. The mean age of breast cancer patients was 49.05±11.70 years (range 25-85 years) and controls was 49.03±11.69 years (range 25-85 years). Breast cancer incidence was higher among individuals more than 40 years of age (79%) compared to those less than 40 years (21%). Of 200 breast cancer patients, 23 had stage I, 104 had stage II, 54 had stage III and 19 had stage IV tumors. There was no significant difference in gender, age, habitat, diet and menstrual history of breast cancer patients and control individuals ($p>0.05$) (Table 1).

**Table 2. Genotype and Allele Frequencies of *HIF-1α* Polymorphisms in Breast Cancer Patients and Controls**

| Variant | | | Patients n(%) | Controls n(%) | OR(95% CI) | $\chi^2$ value | p value |
|---|---|---|---|---|---|---|---|
| g.C111A | Genotype | CC | 200 (100) | 198 (99.0) | - | | |
| | | CA | - | 2 (1.0) | - | NC | NC |
| | | AA | - | - | - | | |
| | | Allele | | | | | |
| | | C | 400 (100) | 398 (99.5) | - | | |
| | | A | - | 2 (0.5) | - | | |
| g.C1772T | Genotype | CC | 152 (76.0) | 149 (74.5) | 1(Reference) | | |
| (rs11549465) | | CT | 38 (19.0) | 42 (21.0) | 0.89(0.54-1.45) | 0.23 | 0.63 |
| | | TT | 10 (5.0) | 9 (4.5) | 1.09(0.43-2.76) | 0.03 | 0.86 |
| | | Allele | | | | | |
| | | C | 342 (85.5) | 340 (85.0) | 1(Reference) | 0.04 | 0.84 |
| | | T | 58 (14.5) | 60 (15.0) | 0.96(0.65-1.42) | | |
| g.G1790A | Genotype | GG | 200 (100) | 200 (100) | - | NC | NC |
| (rs11549467) | | GA | - | - | - | | |
| | | AA | - | - | - | | |
| | | Allele | | | | | |
| | | G | 400 (100) | 400 (100) | - | | |
| | | A | - | - | | | |

*NC: Not calculated; OR: odds ratio; CI: Confidence intervals

**Table 3. Association Analyses of *HIF-1α* g.C1772T Polymorphism with Breast Cancer Risk**

| Genetic Model | | OR(95% CI) | p value |
|---|---|---|---|
| Dominant model | CT+TT *vs* CC | 0.92(0.59-1.45) | 0.73 |
| Over dominant model | CT *vs* CC+TT | 0.88(0.54-1.44) | 0.62 |
| Recessive model | TT *vs* CC+CT | 1.12(0.44-2.81) | 0.81 |
| Homozygous codominant | TT *vs* CC | 1.09(0.43-2.76) | 0.86 |
| Heterozygous codominant | CT *vs* CC | 0.89(0.54-1.45) | 0.63 |
| Allele contrast | T *vs* C | 0.96(0.65-1.42) | 0.84 |

*OR: odds ratio; CI: Confidence intervals

*Association between HIF-1α polymorphisms and breast cancer risk*

The genotype and allele frequencies of g.C111A, g.C1772T and g.G1790A polymorphisms of *HIF-1α* in the patients and controls are shown in the Table 2. The CC and CA genotype frequency of *HIF-1α* g.C111A polymorphism was 100 *vs* 99% and 0 *vs* 1% in breast cancer patients and healthy controls. AA genotype of g.C111A polymorphism was observed neither in patients nor in control subjects. For g.C1772T polymorphism, the frequency of CC, CT and TT genotype was 76 *vs* 74.5%, 19 *vs* 21% and 5 *vs* 4.5% in breast cancer patients and control individuals respectively. There was no significant difference in genotype and allele frequencies of *HIF-1α* g.C1772T polymorphism between cases and control individuals (p>0.05). For g.G1790A genotypes, all patients and controls had GG genotype; GA and AA genotype was not observed in patients and control individuals. Analyses of various genetic models (Table 3) showed no association of *HIF-1α* g.C1772T polymorphism with breast cancer risk in the studied subjects (p>0.05).

We stratified the study subjects to investigate the relationship of *HIF-1α* g.C1772T polymorphisms with age, menopausal status, habitat, habit and tumor stage of breast cancer patients and observed significant difference in genotype distribution of CC and combined CT+TT genotypes of *HIF-1α* g.C1772T in vegetarian and non vegetarian breast cancer patients (p=0.02) (Data not shown).

## Discussion

Breast cancer is a heterogeneous disease encompassing multiple sub groups with different molecular signatures, prognosis, and responses to therapies and involves lymphangiogenesis (Sorlie et al., 2001; Schoppmann et al., 2002). The presence of hypoxic lesions in solid tumors is associated with a more aggressive tumor phenotype, resistance to radiation therapy and chemotherapy and poor survival (Pouyssegur et al., 2006). *HIF-1α*, the key regulator of hypoxia, regulates gene expression in critical pathways involved in tumor growth and metastases (Bos et al., 2001) and serves as an attractive therapeutic target (Poon et al., 2009). In the present case-control study, we assessed the relationship of g.C111A, g.C1772T and g.G1790A polymorphisms of *HIF-1α* with breast cancer risk.

The c.C111A polymorphism of *HIF-1α* has been identified in the bHLH domain of *HIF-1α*. The bHLH-PAS domain containing amino acids 12-298 are required for dimerization with HIF-1β and binding to the hypoxia response element (HRE) (Jiang et al., 1996). In our study we did not found any association of c.C111A polymorphism with breast cancer risk as variant allele was completely absent in patients and only 0.5% of the controls carried the A allele. Similar to our findings, A allele was not previously observed in breast (Apaydin et al., 2008) and ovarian, cervical and endometrial cancer (Konac et al., 2007). However, Naidu et al. observed a very low frequency of A allele in both breast cancer patients and controls (0.4 *vs* 0.2%) in Malaysian population (Naidu et al., 2009).

Polymorphism g.C1772T (p.P582S) causes activation of *HIF-1α* as a gain of function mechanism driven by stabilization of *HIF-1α* mRNA (Vainrib et al., 2012). Meta-analysis has revealed *HIF-1α* g.C1772T polymorphism as a risk factor of cancer in females in Asian population (He et al., 2013) and it can also increase the risk of cancer metastasis (Zhang et al., 2013). In recent meta-analysis, T allele of g.C1772T polymorphism has been significantly associated with increased risk of cancer in Asians rather than Caucasians (Wu et al., 2014). An association of CT genotype of g.C1772T polymorphism has been reported with large tumor size in esophageal squamous cell carcinoma (Ling et al., 2005) and with more severe ulcerative growth pattern in colorectal adenocarcinoma (Fransen et al., 2006). In contrast to our findings, higher frequency of CT genotype has been reported in patients with breast (Naidu et al., 2009), prostate (Chau et al., 2005; Foley et al., 2009), pancreatic cancer (Wang et al., 2011) and glioma (Xu et al., 2011). For g.C1772T polymorphism, we did not observe any significant differences in genotype and allele distribution between breast cancer patients and controls. Similar to the present study, no significant association of g.C1772T polymorphism was observed in the Turkish (Apaydin et al., 2008), Korean (Kim et al., 2008) and Greek (Zagouri et al., 2012) breast cancer patients. Significant association between Ser/Ser genotype at codon 582 and breast cancer risk has been reported among women with larger tumor size or without lymph node involvement (Lee et al., 2008).

For g.G1790A polymorphism, we did not observe GA and AA genotype in either the breast cancer patients or controls. Thus, no significant association of g.G1790A polymorphism was observed in the present study similar to previous reports for breast cancer (Apaydin et al., 2008; Kim et al., 2008; Naidu et al., 2009). Increased frequency of A allele has been reported in renal (Ollerenshaw et al., 2004), gastric (Li et al., 2009), oral (Munnoz-Guerra et al., 2009), hepatocellular (Hsiao et al., 2010) and pancreatic cancer (Wang et al., 2011). In pancreatic cancer, g.G1790A has been associated with greater amount of tumor-produced *HIF-1α* and bigger tumor volumes indicating its role in carcinogenesis and cancer progression (Wang et al., 2011). Recent meta-analysis showed a significant association between A allele of g.G1790A polymorphism and increased cancer risk in pancreatic, lung, renal, head and neck cancer, but not in breast and prostate cancer (Zhou et al., 2014). They demonstrated that cancers of different sites are exposed to different micro-environmental factors that can regulate or influence the gene expression profiles. It has been reported

that different tissues have different expression profiles of *HIF-1α*, thus the same polymorphism may play different role in different tissue (Ribeiro et al., 2009; Hanahan and Weinberg, 2011). Meta-analysis of 39 studies with 10,841 cases and 14,682 controls documented an association of g.C1772T and g.G1790A polymorphisms of *HIF-1α* with increased cancer risk and suggested that *HIF-1α* polymorphism could be a potential marker for both cancer risk and cancer prognosis (Hu et al., 2014).

For *HIF-1α* g.C1772T polymorphism, unlike the significant association with increased risk of cancer reported in Asians rather than Caucasians (Wu et al., 2014) the results of the present study were similar to reports in Turkish (Apaydin et al., 2008) and Greek (Zagouri et al., 2012) breast cancer patients. This could be attributed to several factors such as heterogeneous ethnic background, and genetic factors that predispose to breast cancer. The population in Amritsar, North-West India has a racial mixture of Indo-Scythian and Caucasian racial elements (Bhasin et al., 1992). Due to population diversity within India, other populations should be screened for *HIF-1α* polymorphisms to elucidate their role in breast cancer pathogenesis.

In conclusion, we did not observe association of any of the studied *HIF-1α* polymorphisms with the breast cancer risk in patients from Punjab state of North-West India.

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