

Research Article

GSTP1 Ile105Val and GPX1 Pro198Leu Polymorphisms and Their Association with Response to Radiotherapy in Nasopharyngeal Carcinoma Patients

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Abstract

Objectives: Radio-resistance poses a major challenge in nasopharyngeal carcinoma (NPC) treatment. Due to individual variations in radio-sensitivity, biomarkers are needed to tailor radiation treatment. Within this frame, the identification of series of genetic signatures mainly SNPs for NPC patients treated with radiotherapy may help to predict treatment outcome and deliver personalized therapy. The aim of this study was to evaluate the possible association between the GSTP1 Ile105Val and GPX1 Pro198Leu polymorphisms and response to radiotherapy in NPC patients.

Methods: From September 2016 to October 2018, a total of 101 patients with confirmed NPC, recruited at Mohammed IV Center for Treatment of Cancer of Casablanca, underwent radiotherapy. DNA was extracted from peripheral blood. Genotyping of the GPX1 Pro198Leu and GPX1 Val105Leu polymorphisms was carried out by PCR amplification and DNA sequencing. SPSS was used to analyse the association of GSTP1 and GPX1 genotypes with clinico-pathological features and response to radiotherapy.

Results: The genotyping data revealed the presence of only two genotypes namely Pro/Pro (57.4%) and Pro/Leu (40.6%) for GPX1 gene. The allelic frequencies of C and T alleles were 78.7% and 21.3% respectively. For GSTP gene, the homozygous genotypes Val/Val and Leu/Leu were detected in 35.6% and 12.9% of patients respectively. The heterozygous genotype Val/Leu prevailed (51.5%). Allelic frequencies showed the presence of the two alleles A and G in 57.1% and 42.9% patients respectively. Statistical analysis failed to find any significant association between GSTP Val105Ile and GPX1 Pro198Leu genetic polymorphisms and socio-demographic and clinico-pathological features as well as response to radiotherapy ($p > 0.05$).

Conclusion: Further research is warranted on the potential role of SNPs within antioxidant genes in radiotherapy response and to identify reliable predictive and non-invasive biomarkers for radio-resistance among NPC patients for personalised therapies.

Keywords: Nasopharyngeal carcinoma, genetic polymorphisms, GSTP1, GPX1, radio-resistance, prognosis

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Nasopharyngeal carcinoma (NPC) is a malignant epithelial neoplasm arising from the epithelial lining of the nasopharynx and poses a public health problem in many countries in Southeast Asia and north Africa. In Morocco, like other countries in the Mediterranean basin, the incidence of NPC is moderate, at 3-8 per 100 000 people.^[1, 2]

NPC is widely known for its multifactorial aetiology, including Epstein–Barr virus (EBV) infection, environmental factors and genetic susceptibility.^[3]

Scientific evidence has shown that NPC is highly sensitive to ionizing radiation. As such, radiotherapy (RT) is the main and the most effective treatment of NPC.^[4, 5] However, the main problem faced by clinicians is the growing number of radio-resistance, affecting the treatment outcome and reducing the overall survival (OS) of NPC patients, which suggest the involvement of genetic susceptibility in this radio-resistance.^[6] Radio-resistance is a complex biological process associated with abnormal DNA damage response, apoptosis, autophagy, gene mutations, cell cycle checkpoint and deregulated signalling pathways which hampered the success of RT.^[7] It's widely accepted that radiotherapy acts directly on cellular components through the ionizing radiations and indirectly through generated reactive oxygen species (ROS) in the cell matrix.^[8] Hence, persistence of ROS in the NPC environment will be of a great interest to produce DNA damage and induce apoptosis. Conversely, rapid detoxification and ROS elimination will be associated with a decrease RT efficacy.

Many genetic studies have been conducted and showed that Pi-class glutathione S-transferase (GSTP1) and glutathione peroxidase1 (GPX1) are the most proteins involved in cell detoxification and xenobiotic elimination. These two proteins play a key role in preventing the accumulation of xenobiotics in cells, reducing their damage and the occurrence of various diseases.

Growing interest was given to investigate the role of GSTP1 and GPX1 and their involvement in cancer development and resistance. GSTP1 enzyme, encoded by the GST pi gene, is an isozyme widely reported to be expressed in human epithelial tissues,^[9] that plays an important regulatory role in detoxification and anti-oxidative damage. GSTP1 is also involved in maintaining cell integrity and protecting DNA from genotoxic and cell-damaging molecules by catalysing the detoxification of reactive electrophilic compounds.^[10] Large studies were conducted on the association between genetic polymorphisms in *GSTP1* gene and cancer development, including NPC, and have focused on some specific polymorphisms like rs1695 (Ile105Val) and rs1138272 (Ala114Val) that alter the catalytic activity of the protein. Indeed, polymorphisms in *GSTP1* gene have been

reported to be associated with bladder and testicular cancers,^[11] head and neck cancers,^[12] hepatocarcinoma,^[13] lung cancer,^[14] oral carcinoma^[15] and breast cancer.^[16, 17]

GPX1 is a selenium-dependent enzyme highly involved in cell antioxidant defence by protecting cells and their environments from oxidative damage. GPX1, encoded by *GPX1* gene, is able to eliminate the hydrogen peroxide (H₂O₂) through its reduction into water.^[18] In *GPX1* gene, a wide interest is given to rs1050450C>T polymorphism, resulting on proline (Pro) to leucine (Leu) amino acid substitution at codon 198 (Pro198Leu), close to the C-terminus of the protein.^[19] GPX1 Pro198Leu SNP, has been shown to decrease the enzymatic activity of glutathione peroxidase in the cells expressing the mutant protein, which further can increase the oxidative damage.^[20] GPX1 Pro198Leu polymorphism has been shown to be associated with colorectal and gastrointestinal cancers in relationship with exposure at various environmental factors.^[21, 22] A recent meta-analysis has reported a positive correlation between GPX1 Pro198Leu SNP and the development of bladder, head and neck and brain cancers.^[23]

There are limited data addressing the association of genetic polymorphisms in *GSTP1* and *GPX1* genes with tumour response to radiotherapy and/or chemotherapy. Accordingly, an association between *GSTP1* polymorphism and treatment outcome was reported in breast cancer,^[24] bladder cancer^[25] and NPC,^[26] providing accumulating evidence of the role of genetic variants in predicting response to radio and/or chemotherapy in cancer patients. Data regarding genetic background of radio-resistance-related SNPs in NPC are scarce. So, we designed this study to investigate the association of Pro198Leu and Ile105Val polymorphisms in detoxification genes namely *GPX1* and *GSTP1* with response to radiotherapy in NPC patients and thus their potential use as biomarkers for better management of NPC in Morocco.

Methods

Study Design

A total of 101 histologically confirmed NPC patients were recruited at Mohammed IV Center for Treatment of Cancer of Casablanca, between September 2016 and October 2018. Patients with previous cancer and those who had previous chemotherapy or radiotherapy treatments were excluded from this study.

A questionnaire was administrated to each patient to collect information regarding the socio-economic status (age, sex, familial history of cancer, tobacco habits, alcohol consumption and childhood habitat). Clinical data including

histological type and clinical stage were retrieved from their medical records.

Patients were given a median dose of 50 Gy in the nasopharyngeal tumour area and 66 Gy in the involved lymph nodes. A daily fraction of 2 Gy was delivered five times per week and the treatment was completed within 5 to 7 weeks. For patients with an advanced stage of the disease, neoadjuvant chemotherapy was administered using different regimens based on cisplatin, anthracycline and fluorouracil.

Clinical staging of NPC was determined according to the 7th edition of the International Union Against Cancer (IUAC) and American Joint Committee on Cancer (AJCC) staging system.^[27] The follow-up was done at 3 month intervals for the first two years and six monthly thereafter. All patients were subjected to peripheral blood sampling before initiation of the treatment for plasmatic cell-free DNA extraction. The study protocol was approved by the Ethics Committee of Ibn Rochd Hospital of Casablanca, Morocco; and a written informed consent was obtained from each patient before its enrolment.

DNA Extraction

Genomic DNA was extracted from leukocyte nuclei using phenol chloroform DNA extraction method. DNA concentration and purity were assayed using the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). DNA was used immediately for PCR amplification or stored at -20°C until use.

GPX1 Pro198Leu and GSTP1 Ile105Val Genotyping

Pro198Leu and Ile105Val polymorphisms screening were carried out by PCR amplification and DNA sequencing. For GPX1Pro198Leu, primers flanking a region in exon 2 of *GPX1* gene containing the SNP rs1050450 C>T were used. For GSTP1 Ile105Val, primers flanking a region in exon 6 of *GSTP1* gene harboring the SNP rs1695 A>G were used. The primers sequences of the two SNPs and their characteristics are shown in Table 1.

PCR amplification was performed in a total volume of 25µl, containing 1X PCR buffer, 200µM of each dNTP, 200nM of each primer, 1.5 mM MgCl₂, 0.5U Platinum Taq DNA poly-

merase (Invitrogen) and 50ng of genomic DNA. The mixtures were first denatured at 94°C for 7 min. Then, 40 cycles of PCR were performed with denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s and primer extension for 30 s at 72°C. At the end of the last cycle, amplicons were incubated at 72°C for 7 min. For each PCR run, a negative control, in which DNA template was omitted from the amplification mixture, was included. GSTP1 and GPX1 fragments' amplification was checked by DNA size separating by electrophoresis on 2% agarose gel and visualization under ultraviolet light.

PCR products were then purified using ExoSAP-IT® (Applied Biosystems) to eliminate the primers and unincorporated remaining dNTPs. Sequencing of purified PCR products was done with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were performed in a final volume of 10µl, containing 1µl of 2.5X Big Dye ready reaction mix v3.1, 10 pmol of forward or reverse primer and 100ng of purified PCR product. Sequencing mixtures were first incubated at 96°C during 1 minute, then followed 25 cycles of denaturation at 96°C for 10s, primer annealing at 50°C for 5s and extension at 60°C for 4 min. The sequencing reaction products were then purified using Sephadex G-50 gel-exclusion chromatography (GE Healthcare Life Sciences) to eliminate the excess of ddNTPs. Direct sequencing of amplified DNA was performed by capillary electrophoresis on an ABI 3130xL Genetic Analyzer (Applied Biosystems). The sequences were analyzed using Sequencing Analysis v5.4 software (Applied Biosystems).

Statistical Analysis

Statistical analyses were performed using Software Package for Social Sciences (SPSS) version 23.0. The differences were considered significant if the p value did not exceed 0.05. Allelic frequency and genotypic frequencies were also calculated using the corresponding formula.

Results

The socio-demographic, clinical and pathological characteristics of all cases are summarized in Table 2. The median age of the recruited NPC patients was 54 years old,

Table 1. Primers Characteristics

Gene	rs no.	SNP	Codon	Change in amino acid	Primer sequences	Amplicon size (Bp)
GPX1	rs1050450	C>T	198	Pro>Leu	5'-CGCCACCGCGCTTATGACCG-3' 5'-GCAGCACTGCAACTGCCAAGCAG-3'	238
GSTP1	rs1695	A>G	105	Val>Ile	5'-ACCCAGGGCTCTATGGGAA-3' 5'-TGAGGGCACAAGAAGCCCT-3'	176

Table 2. Socio-demographic, clinical and pathological characteristics of NPC patients

Parameter	Total cases	Percentage (%)
Gender		
Male	61	60.39
Female	40	39.61
Age		
≤30	23	23.76
>30	78	76.24
Smoking habit		
Smoke	32	31.68
Non-smoke	69	68.32
Alcoholic habit		
Alcoholic	10	9.9
Non-Alcoholic	91	90.1
Tumour stage		
I-II	13	12.87
III-IV	88	87.13
Histological type		
Type I	1	0.99
Type II	7	6.93
Undifferentiated type	90	89.1
Other types	3	2.97
TNM		
Tumoral extension		
T1-T2	24	23.8
T3-T4	77	76.2
Regional lymph node involvement		
N=0	8	7.9
N≥1	93	92.4
Metastasis		
M0	65	75.2
M1	36	24.8

ranging from 12 to 80 years old. In this cohort, males' patients prevailed (60%) with sex ratio of 1.5. Smoking and alcoholic habits concerned 31.68% and 9.9% of the patients respectively. The clinico-pathological tumor characteristics revealed that 87.13% of patients were at advanced stages (III and IV), and most patients exhibited the undifferentiated type (89.1%). TNM staging revealed that 75% of patients were classified in T3-T4 categories and up to 34.6% of patients presented distant metastasis (M1). No involvement of regional lymph nodes was observed in 7.9% of patients.

In this study, we identified SNPs (rs1050450 and rs1695) by PCR-Sequencing within NPC patients.

Genotypes Distribution and Allelic Frequencies

For *GPX1* gene, only two genotypes were detected namely Pro/Pro (57.4%) and Pro/leu (40.6%). The homozygous

genotype (Leu/Leu) of the *GPX1* variant was not found. The allelic frequencies of C and T alleles were 78.7% and 21.3% respectively.

For *GSTP* gene, the heterozygous genotype Val/Leu prevailed (51.5%). The homozygous genotypes Val/Val and Leu/Leu were detected in 35.6% and 12.9% of patients respectively. Allelic frequencies showed the presence of the two alleles A and G in 57.1% and 42.9% patients respectively (Table 3).

Association between SNPs and Socio-Demographic & Clinico-Pathological Parameters

The genotypes distributions of *GPX1* Pro198Leu and *GSTP1* Val105Ile polymorphisms according to socio-demographic and clinico-pathological characteristics of NPC patients are summarized in Tables IV and V respectively. The expressions of genotypes were unrelated to gender, age, and clinical stage. Overall, no association was detected between *GPX1* Pro198Leu genetic variants and any of the studied parameter. Also, Statistical analysis failed to find any significant association between *GSTP1* Val105Ile polymorphism and socio-demographic and clinico-pathologic features.

Among the 101 diagnosed NPC patients, 67.3% (68/101) underwent radiotherapy and among them, 86% had a complete remission. Although C allele was four times more prevalent than T allele for *GPX1* Pro198Leu polymorphism, statistical analysis failed to find any significant association between this polymorphism and radiotherapy response ($p > 0.05$) (Table 4).

For *GSTP1* Ile105Val polymorphism, we noted that both alleles had same frequencies within radio-resistant patients. Despite the prevalence of heterozygous genotypes AG within radiosensitive patients, no significant association was found between this polymorphism and response to radiotherapy ($p > 0.05$) (Table 5).

Table 3. Genotypes distribution and allelic frequencies within NPC patients

Gene/ Genotype	No. Of patients	%	Allele	%
GPX1				
CC	58	57.4	C	78.7
CT	43	42.6	T	21.3
TT	0	0		
GSTP1				
AA	36	35.6	A	57.1
AG	52	51.5	G	42.9
GG	13	12.9		

Table 4. Association between demographic, clinico-pathological data and GPX1 Pro198Leu polymorphism

GPX1 Pro198Leu	N	Genotype				Allele		
		CC (%)	CT(%)	TT(%)	P	C(%)	T(%)	P
Sexe								
Female	40	20 (50.00)	20 (50.00)	0	0.221	60 (75.00)	20 (25.00)	0.317
Male	61	38 (62.29)	23 (37.70)	0		99 (81.14)	23 (18.85)	
Age								
≤ 30	23	12 (52.17)	11 (47.82)	0	0.582	35 (76.08)	11 (23.91)	0.689
> 30	78	46 (58.97)	32 (41.02)	0		124 (79.48)	32 (20.51)	
Clinical stage								
I-II	13	6 (46.15)	7 (53.84)	0	0.417	19 (73.07)	7 (26.92)	0.467
III-IV	87	51 (58.62)	36 (41.37)	0		140 (79.54)	36 (23.70)	
Response to radiotherapy								
RadioS	57	33 (57.89)	24 (42.11)	0	0.814	90 (78.94)	24 (21.06)	0.922
RadioR	11	6 (54.55)	5 (45.45)	0		17 (77.27)	5 (22.63)	

Table 5. Association between demographic, clinico-pathological data and GSTP1 Ile105Val polymorphism.

GSTP1 Ile105Val	N	Genotype				Allele		
		AA (%)	AG (%)	GG (%)	P	A (%)	G (%)	P
Sex								
Female	40	13 (32.5)	19 (47.5)	8 (20)	0.894	45 (60.81)	29 (39.18)	0.91
Male	61	23 (37.70)	33 (54.1)	5 (8.2)		79 (61.71)	49 (38.28)	
Age								
≤ 30	23	7 (30.43)	13 (56.52)	3 (13.04)	0.562	27 (40.90)	19 (28.78)	0.897
> 30	78	29 (37.17)	39 (50.00)	10 (12.82)		97 (62.17)	59 (37.82)	
Clinical Stage								
I-II	13	7 (53.84)	4 (30.76)	2 (15.38)	0.902	18 (69.23)	8 (30.76)	0.793
III-IV	87	28 (32.18)	48 (55.17)	11 (12.64)		104 (60.36)	70 (40.22)	
Response to radiotherapy								
RadioS	57	19 (33.33)	30 (52.63)	8 (12.90)	0.378	88 (65.67)	46 (34.32)	0.666
RadioR	11	3 (27.27)	5 (45.45)	3 (27.27)		11 (50.00)	11 (50.00)	

Discussion

NPC is a malignant disease with skewed ethnic and geographic distribution.^[28] The deep-seated anatomical position of NPC made this malignancy not accessible by surgery and thus the principal treatment approach is radiotherapy. Although NPC is highly radio-sensitive, patients are prone to recur loco-regionally and to develop distant metastasis.^[29]

Radio-resistance hampers the success of treatment of NPC and multiple signalling pathways are involved in this phenomenon.^[30] Hence, deciphering new molecular targets and pathways is essential for enhancing and promoting the radio-sensitivity of NPC patients since clinical TNM staging has limited accuracy in NPC response prediction. Despite progress towards molecular mechanisms underlying radiotherapy response, there are no effective biomarkers for NPC

radio-sensitivity/radio-resistance.^[31] Up to date, there is no standard test available that may reliably predict radio-sensitivity for cancer patients.^[32] Due to individual variations in radio-sensitivity, biomarkers are selected to tailor radiation treatment to NPC. Hence, there is a need to identify genes involved in radio-resistance along with the biological process contributing to the development of radio-resistance.^[31]

Extensive studies reported that some genetic polymorphisms have been implicated in increased susceptibility to NPC.^[33, 34] Of interest, several studies have evaluated the association between GSTP1 c.105A>G and GPX1 c.198C>T polymorphisms and risk of cancer development with type specific effects, however, these studies yielded conflicting results.^[23, 35-41]

By establishing a radio-resistant cell line, Guo et al. identified a panel of candidate's genes including GSTP1 whom

might become a potential biomarker in predicting NPC response to radiotherapy.^[31] In this study, we identified c.105A>G SNP in GSTP1 gene within NPC patients and the results clearly showed that this polymorphism had no relevant effect neither on clinic-pathological features nor with radiotherapy response. Likewise, in a recent study, no association was detected between this polymorphism and primary tumour site, clinico-pathological characteristics, treatment (chemotherapy and/or radiotherapy) and survival time of the head and neck cancer (HNC) patients.^[42] Moreover, a meta-analysis performed by Lang et al. failed to qualify c.105A>G SNP in GSTP1 gene as risk for developing HNC in many studies.^[43]

To the best of our knowledge, no study has investigated the association between this SNP and RT response in NPC. The variant genotype was reported to be linked with CT and RT responses and side effects in different malignancies. Pasqualetti et al have shown that this SNP was associated with different OS in glioblastoma patients treated with RT and temozolamide.^[44] Also, it was reported that this SNP might be a useful biomarker for prediction of clinical benefit and toxicities of platinum-based chemotherapy in Thai epithelial ovarian cancer.^[45] In contrast, Deng et al. found that patients carrying GSTP1 variant genotypes were rather at lower risk of recurrence following an intravesical therapy and thus, suggested that c.105A>G SNP may predict chemotherapy outcome in patients with bladder cancer (BC) and may, therefore, serve as molecular marker to monitor BC recurrence.^[25] Similarly, in a meta-analysis performed by Shen et al. GSTP1 Ile105Val polymorphism was associated with tumour response, progression free survival, and OS in gastric and colorectal cancers after CT.^[46]

Accumulating evidence implicates that GPX1 polymorphism (rs1050450) contributes to tumour susceptibility to cancer; individuals carrying variant Leu allele (Pro/Leu and Leu/Leu) were associated with an increased risk of cancer.^[47, 48] In NPC, the association between became evidenced; Laribi et al. found strong association between GPX1 Pro-198Leu genetic variant and NPC risk. Moreover, this variant increased the risk of progression to regional lymph node metastasis.^[49] Dequanter et al. found that GPX1 low expressing HNSCC were at T3-T4 clinical stages and that GPX1 expression does not influence neither the response to radiotherapy nor the survival.^[50] In contrast, in the present study, the expressions of GPX1 Pro198Leu genotypes in patients with NPC were irrelevant to gender, age, clinical stage and response to RT.

Indeed, studies on the relationships between genetic polymorphisms in radio-resistance related genes and radiotherapy sensitivity in NPC have been performed.^[31, 49]

Ban et al. suggest Wnt/beta-catenin as a novel prognostic factor for NPC patients treated with RT.^[51] More specifically, rs18880481 and rs3864004 polymorphisms in the catenin beta 1 gene (CTNNB1) and rs3755557 polymorphism in the glycogene synthase kinase 3 beta gene (GSK3beta), were significantly associated with a poorer efficacy of RT in NPC patients.^[52] Wang et al. have investigated the relationship between SNPs (G2677T exon 21 and C3435T in exon 26) in human multidrug resistance 1 gene (MDR) and radio-sensitivity and found that patients with 2677G-3435C haplotype had better response to radiotherapy compared to other haplotypes.^[53] Very recently, inflammation-associated gene SNPs were found to be associated with increased radiotherapy sensitivity of HNSCC patients.^[54] Likewise, Seibold et al. reported that variants in oxidative stress-related genes may affect radiotherapy response.^[55] However, in NPC, the relationship between SNPs in detoxification related genes and the response of NPC patients to radiotherapy has not been reported.

Plenty of studies were performed to access the potential role of GPX1 and GSTP1 SNPs in the risk of Cardiovascular diseases^[56] and solid tumours^[57] but studies on their contribution to predict (chemo)radiotherapy treatment outcomes in NPC patients are very scarce. Treatment outcome is difficult to set; it is hampered by heterogeneity of tumours. Indeed, patients with same clinical stages, according to the TMN staging system, have different outcomes albeit receiving similar treatments. Recently, accumulated knowledge on tumour behaviour as well as response to cancer treatment has led to emergence of radiogenomics which links genetic variation with response to radiotherapy to improve decision making and thus patient outcomes.^[58]

Overall, the present study failed to statistically establish the associations between the well characterized functional GSTP1 c.105A>G and GPX1 c.198C>T polymorphisms within NPC patients and response to radiotherapy. Further studies with larger sample size and targeting additional polymorphisms may better demonstrate the interaction of GSTP1 & GPX1 germline polymorphisms as well different haplotypes and response to the radiotherapy and then select potential candidate biomarkers to predict NPC response to radiotherapy for personalized therapeutic strategies in the future.

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