

**GSTM1, GSTT1, GSTP1 and CYP1A1 genetic polymorphisms and susceptibility to esophageal cancer in a French population: Different pattern of squamous cell carcinoma and adenocarcinoma**

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**Abstract**

AIM: To evaluate the association between CYP1A1 and GSTS genetic polymorphisms and susceptibility to esophageal squamous cell carcinoma (SCC) and esophageal adenocarcinoma (ADC) in a high risk area of northwest of France.

METHODS: A case-control study was conducted to investigate the genetic polymorphisms of these enzymes (CYP1A1*2C and GSTP1 exon 7 Val alleles, GSTM1*2/*2 and GSTT1*2/*2 null genotypes). A total of 79 esophageal cancer cases and 130 controls were recruited.

RESULTS: GSTM1*2/*2 and CYP1A1*1A/*2C genotype frequencies were higher among squamous cell carcinomas at a level close to statistical significance (OR = 1.83, 95% CI 0.88-3.83, P = 0.11; OR = 3.03, 95% CI 0.93-9.90, P = 0.07, respectively). For GSTP1 polymorphism, no difference was found between controls and cases, whatever their histological status. Lower frequency of GSTT1 deletion was observed in ADC group compared to controls with a statistically significant difference (OR = 13.31, 95% CI 1.66-106.92, P<0.01).

CONCLUSION: In SCC, our results are consistent with the strong association of this kind of tumour with tobacco exposure. In ADC, our results suggest 3 distinct hypotheses: (1) activation of exogenous procarcinogens, such as small halogenated compounds by GSTT1; (2) contribution of GSTT1 to the inflammatory response of esophageal mucosa, which is known to be a strong risk factor for ADC, possibly through leukotriene synthesis; (3) higher sensitivity to the inflammatory process associated with intracellular depletion of glutathione.

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**INTRODUCTION**

One of the highest incidences of esophageal cancer in Europe is observed in the Northwest of France[1-4]. There are two predominant histological forms of this cancer: squamous cell carcinoma (SCC) and adenocarcinoma (ADC)[4,5]. Recent epidemiological observations showed an important decrease in the incidence of SCC whilst ADC was slightly increased[4,6]. In Western countries, smoking tobacco and drinking alcohol are the main risk factors for SCC. For ADC, exogenous risk factors are not well known. A link was found between this pathology, esophageal reflux and Barrett’s esophagus[5].

Tobacco smoke contains many carcinogens such as polycyclic aromatic hydrocarbons (PAH) and N-nitrosamines that can be activated or deactivated by phase I (cytochromes P-450) and phase II enzymes (glutathione S-transferases). Cytochromes P450 (CYP) are a widely expressed enzyme family, some members of which present genetic polymorphisms (e.g. CYP1A1, 2E1, 2D6). CYP1A1 is expressed in esophageal mucosa, which means that activation of tobacco carcinogens can happen *in situ*[6]. Benzo[a]pyrene is activated by CYP1A1 to diol-epoxide, which is a reactive and carcinogenic product. Four main genetic polymorphisms are described for CYP1A1. One of the most studied is Ile/Val polymorphism in exon 7 (CYP1A1*2C allele). One study reported that Val-type could be associated with a higher aryl hydrocarbon hydroxylase activity[7]. Many studies have reported the association of CYP1A1 polymorphisms with lung cancer[8,9], particularly with SCC of the lung[8,9]. The level of DNA adducts was found to be linked to CYP1A1*2C polymorphism[8]. All these results suggest that susceptibility to tobacco-related cancers could be modified by CYP1A1 polymorphisms.

The glutathione S-transferases are a family of phase II enzymes, which catalyse the conjugation of many endogenous and exogenous electrophilic compounds to glutathione. GSTM1 and GSTP1 are able to detoxify benzo[alpha]pyrene diol-epoxide[10], whereas GSTT1 can conjugate oxidised lipids and halogenated compounds[11]. Both GSTM1, P1 and T1 are expressed in esophageal mucosa[12,13]. GSTP1 is the mainly expressed GST in this tissue[14]. GSTP1 presents a substitution polymorphism in exon 7 that results in a substitution of Ile by Val at amino acid position 104[15]. Val variants were found to have a lower activity[16]. GSTT1 deletion of chromosome 11 is a frequent polymorphism[17]. Both GSTM1, P1 and T1 are expressed in esophageal mucosa[17,18]. GSTP1 is the mainly expressed GST in this tissue[19]. GSTP1 presents a substitution polymorphism in exon 7 that results in a substitution of Ile by Val at amino acid position 104[16]. Val variants were found to have a lower activity towards 1-chloro-2, 4-dinitrobenzene[17]. GSTM1 and GSTT1 present deletion polymorphisms (GSTM1*2/*2 and GSTT1*2/*2), which are currently at about 50% and 20% among Caucasians, respectively[18,19]. GSTM1*2/*2 polymorphism has been found to increase the frequency of chromosome aberrations after tobacco-specific N-nitrosamine exposure *in vitro*[20]. Many studies have shown that this deletion increases the susceptibility conferred by the CYP1A1*2C allele for tobacco-associated cancer[21]. While GSTT1*2/*2 genotypes have not been clearly...
associated with susceptibility to tobacco-linked cancers, an interaction with GSTM1*/2/*2 has often been found[22,23].

The aim of our work was to evaluate the susceptibility conferred by CYP1A1 and GSTs genetic polymorphisms to SCC and ADC of esophagus in a high risk area.

MATERIALS AND METHODS

Controls and cases were from the geographic area of Basse-Normandie, France. Patients were recruited after endoscopic and histologic diagnosis of primary esophageal cancer. All cases were newly diagnosed and previously untreated patients. Controls were required to be free of any chronic diseases, having no cancer history and living in Basse-Normandie. They were matched with cases in sex and age. Alcohol and tobacco consumption were also evaluated during the recruitment of cases and controls by means of a questionnaire.

The research protocol was approved by the Comité Consultatif pour la Protection des Personnes dans la Recherche Biomédical en Basse-Normandie. A 20 mL sample of venous blood was taken and DNA extraction was performed by phenol/chloroform method.

The primer sequences and product sizes of each gene amplification are shown in Table 1. GSTM1 and GSTTI multiplex PCR was performed according to the Lin et al. method[8], with some modifications. A final mixture volume of 25 µL was prepared containing 0.100 µg of DNA, 0.25 µmol/L of dNTP, 0.4 µmol/L of primer for GSTM1, 0.8 µmol/L of primer GSTTI, 0.8 µmol/L of primer albumin, 5 µL of 10x buffer, 2 mmol/L of MgCl2 and 0.5 U per sample of DNA Gold Taq polymerase (Applied Biosystem, Courtaboeuf, France). The first step was performed for 15 min at 95°C followed by 35 cycles: at 94°C for 1 min (denaturation), at 58°C for 1 min (annealing), at 72°C for 1 min (elongation). PCR ended a final extension for 10 min at 72°C. PCR products were visualised on 20 g/L agarose gel with ethidium bromide staining.

| Table 1 Primer sequences and length of PCR products |
|----------------------------------------------------|
| **Gene** | **Primer sequence** | **Size of PCR product (bp)** |
| GSTM1 | 5'-GAACCTCCCTGAAAAAGCTAAAGGC-3' | 219 |
| | 5'-GGTGGGCTTCATAATAGCCTG-3' | 1998[8] |
| GSTTI | 5'-TTCTTACTGGTCCTCATACTTC-3' | 459 |
| | 5'-TCCAGGATCATGGCACGA-3' | 1998[8] |
| Albumin | 5'-GGGCTGTCATCAAGACTCTC-3' | 350 |
| | 5'-GCCTTAAAGAGAAAAAGACTC-3' | |
| GSTP1 | 5'-GCCCTCTGCTAACAAGTCC-3' | 1997[14] |
| | 5'-GCCCTCTGCTAACAAGTCC-3' | 1997[14] |
| CYP1A1 | 5'-GGCTGAGCAATCTGACCCTA-3' | 206 |
| | 5'-GGCTGAGCAATCTGACCCTA-3' | 206 |

GSTP1 PCR restriction fragment length polymorphism (rflp) was performed using a method adopted by Harries et al.[16] with slight modifications. The final mixture (40 µL) was prepared containing 0.100-0.500 µg of DNA, 0.25 µmol/L of dNTP, 0.25 µmol/L of each of the primers, 1.25 µmol/L of MgCl2, 4 µL of 10x buffer, 4 µL of DMSO, 1.5 U of Taq polymerase (Eurobio, Les Ulis, France). Briefly, the samples were denatured at 94°C for 5 min and submitted to 30 cycles of amplification as follows: for 30 s at 94°C (denaturation), for 30 s at 55°C (annealing), for 30 s at 72°C (extension) and a final extension at 72°C for 5 min. PCR product of 12 µL was digested by 5 U Alw26 I restriction enzyme (Eurogentec, Seraing, Belgium) for 12 h at 37°C. Migration was performed on low melting 40 g/L agarose gel (Eurobio, Les Ulis, France), stained with ethidium bromide, in order to separate the 85 and 91 bp fragments.

CYP1A1*2C polymorphism was determined by PCR-RFLP as previously described[8].

Each PCR analysis was performed twice in double blind.

Statistical analysis

Chi-square test and P value estimation were performed using Stata software (STATA Corporation, college Station, TX). Odds ratio was also evaluated using StataO software and adjusted for age, sex and histological type.

RESULTS

The populations of controls and cases are described in Table 2. The patient group consisted of 52 SCCs and 27 ADCs. The mean ages for cases and controls were 62 and 56 years respectively. Unfortunately, we obtained tobacco and alcohol exposure data for only 48 cases. This was insufficient to allow us to study interaction between exposure and polymorphisms. As it could be expected, the vast majority of SCCs were smokers (93%, all with more than 20 years of tobacco consumption) and heavy drinkers (86% drinking more than 229 g/wk). Fewer ADCs were smokers (78%, of which 67% with more than 20 years of tobacco consumption) and only 50% were heavy drinkers (Table 2).

| Table 2 Description of control and case populations |
|----------------------------------------------------|
| **Table 2** Description of control and case populations |
| **Control n (%)** | **Case n (%)** | **SCC n (%)** | **ADC n (%)** |
| Male | 87 (0.67) | 69 (0.87) | 44 (0.85) | 25 (0.93) |
| Female | 43 (0.33) | 10 (0.13) | 8 (0.15) | 2 (0.07) |
| Mean age (yr) | 56 [19; 87] | 62 [40; 85] | 60 [40; 78] | 66 [51; 85] |
| Tobacco duration | (years of smoking) | | | |
| Non-smokers | 66 (0.66) | 6 (0.13) | 2 (0.07) | 4 (0.22) |
| 1-19 | 13 (0.13) | 2 (0.04) | 0 (-) | 2 (0.11) |
| +20 | 21 (0.21) | 40 (0.83) | 28 (0.93) | 12 (0.67) |
| Alcohol consumption | (g of ethanol per week) | | | |
| 0-228 | 11 (0.64) | 13 (0.28) | 4 (0.14) | 9 (0.50) |
| 228.5 -> +470 | 6 (0.36) | 35 (0.72) | 26 (0.86) | 9 (0.50) |

1Tobacco duration (year); 2Data were available for 77% of controls, 61% of cases, including 58% of SCC and 67% of ADC; 3Alcohol consumption (gram of ethanol per week); 4Data for alcohol consumption were available for only 13% of controls, 61% of cases, including 58% of SCC and 67% of ADC.

Frequencies of the different genetic polymorphisms in the control group were 0.06 for CYP1A1*1A/*2C (no homozygous 2C/*2 subject was found), 0.45 and 0.07 for Ile/Val and Val/Val GSTP1 genotypes, 0.49 for GSTM1*2/*2 and 0.26 for GSTTI*2/*2 (Tables 3, 4).

A high frequency of CYP1A1*1A/*2C genotype was found in SCC cancer patients (Table 3). However, the difference did not reach statistical significance (with a P value of 0.06). The ADC patient group did not show any significant difference compared to the control group.

GSTM1*2/*2 genotype (GST M1 null) was increased among the cases compared to the controls, particularly among SCC patients (Table 4), but this difference was not statistically significant (OR = 1.83; 95% CI = 0.88-3.83). The distribution of GSTM1*2/*2 genotype among ADCs did not differ from the controls.

The frequency of GSTTI*2/*2 genotype (GSTTI null) was not different between cases and controls (Table 5). However, the ADC group showed a greatly decreased frequency of GSTTI*2/*2 genotype (4%) compared to the control population (26%) and SCCs (29%) (OR = 13.31, 95% CI = 1.66-106.92).

Distribution of the GSTP1 genotype did not differ between SCC, ADC and control groups (Table 6).
Table 3 Repartition of CYP1A1 genotypes among controls and cases

|        | CYP1A1*1A/*1A | CYP1A1*1A/*2C | OR$^1$ | 95% CI       |
|--------|---------------|---------------|--------|--------------|
| Controls | 107           | 101           | 6      | (6)          |
| Cases   | 70            | 61            | 9      | (13)         |
| SCC     | 47            | 40            | 7      | (15)         |
| ADC     | 23            | 21            | 2      | (9)          |

$^1$Adjusted OR for age and sex; $^2$Comparison of CYP1A1*1A/*2C genotype repartition in controls vs all cases; $^3$Comparison of CYP1A1*1A/*2C genotype repartition in controls vs SCCs; $^4$Comparison of CYP1A1*1A/*2C genotype repartition in controls vs ADCs; $^p = 0.067$.

Table 4 Repartition of GSTM1 genotypes among controls and cases

|        | GSTM1*2/*2 | OR$^1$ | 95% CI       |
|--------|------------|--------|--------------|
| Controls | 120        | 59     | (49)         |
| Cases   | 68         | 39     | (57)         |
| SCC     | 43         | 27     | (63)         |
| ADC     | 25         | 12     | (48)         |

$^1$Adjusted OR for age and sex; $^2$Comparison of GSTM1*2/*2 genotype repartition in controls vs all cases; $^3$Comparison of GSTM1*2/*2 genotype repartition in controls vs SCCs; $^4$Comparison of GSTM1*2/*2 genotype repartition in controls vs ADCs; $^p = 0.108$.

Table 5 Repartition of GSTT1 genotypes among controls and cases

|        | GSTT1*2/*2 | OR$^1$ | 95% CI       |
|--------|------------|--------|--------------|
| Controls | 115        | 30     | (26)         |
| Cases   | 70         | 14     | (20)         |
| SCC     | 44         | 13     | (29)         |
| ADC     | 26         | 1      | (4)          |

$^1$Adjusted OR for age and sex; $^2$Comparison of GSTT1*2/*2 genotype repartition in controls vs all cases; $^3$Comparison of GSTT1*2/*2 genotype repartition in controls vs SCCs; $^4$Comparison of GSTT1*2/*2 genotype repartition in controls vs ADCs; $^p < 0.05$.

Table 6 Repartition of GSTP1 genotypes among controls and cases

|        | GSTP1 | OR$^1$ | 95% CI       |
|--------|-------|--------|--------------|
| Controls | 124   | 59     | (48)         |
| Cases   | 70    | 31     | (44)         |
| SCC     | 45    | 21     | (47)         |
| ADC     | 25    | 10     | (40)         |

$^1$Adjusted OR for age and sex; $^2$Ile/Val and Val/Val genotypes were compared to Ile/Ile genotype; $^3$Comparison of GSTP1 Ile/Ile and Val/Val genotype repartition in controls versus all cases; $^4$Comparison of GSTP1 Ile/Ile and Val/Val genotype repartition in controls vs SCCs; $^5$Comparison of GSTP1 Ile/Ile and Val/Val genotype repartition in controls vs ADCs.

DISCUSSION

Esophageal cancer presents a very variable incidence in different regions and ethnic groups. In France, different levels of environmental exposure to carcinogens could not fully explain this high variability,[25-27] a fact which suggests a genetic susceptibility. Many epidemiological studies have established that exposure to tobacco smoke and alcohol is a major risk factor for SCC in Western countries, whereas ADC is not strongly linked to exogenous factors. As far as we know, only one study concerning the genetic susceptibility to esophageal cancer was performed among Caucasians[28]. Moreover, the cases for this study were recruited in a low risk area in Europe.

The repartition of different polymorphisms in our control group agrees with available data for a Caucasian population.[16,18,19,20] Recently, frequencies of these polymorphisms among a healthy population were evaluated and published by International Collaborative Study on Genetic Susceptibility to Environmental Carcinogens (GSEC)[18].

Among SCC cases, CYP1A1*2A/*2C frequency was increased when compared to controls and adjusted OR was 3.03 (95% CI 0.93-9.90); however this result was not statistically significant ($P = 0.067$). The deletion of GSTM1 gene was also more frequent among SCC cases when compared to controls (63% and 49% respectively, OR = 1.83; 0.88-3.83). But this result was also not statistically significant ($P = 0.108$). CYP1A1*2A/*2C and GSTM1*2/*2 genotypes were found to increase the risk of SCC in a previous study in an Asian population, particularly among cases with higher tobacco consumption. However, some studies did not find CYP1A1 and GSTM1 gene polymorphisms to be related to SCC. No association was found between other genetic polymorphisms studied (GSTT1, GSTP1) and esophageal SCC, which is in accordance with the data in literature.[24,30,31] It should be emphasized that, concerning GSTT1, our study is the first report about a Caucasian population.

No differences were found among ADC cases regarding the
frequencies of \( \text{CYP1A1}, \text{GSTM1} \) and \( \text{GSTP1} \) polymorphisms when compared to controls. This observation is in accordance with the weak association of tobacco smoke, alcohol consumption and ADC. In the ADC group, an unexpected protective effect of \( \text{GSTT1} \) deletion was found (OR = 13.31; 95% CI 1.66-106.92). Such results have been previously described for other sites such as renal or prostate carcinoma\(^2,3,5\). It is well known that the risk of renal carcinoma is increased by exposure to small halogenated compounds such as dichloromethane or trichloroethylene. Activation of these compounds in electrophilic species implies \( \text{GSTT1} \), which could explain these results. However, to our knowledge, no studies have demonstrated a role of small halogenated compounds in esophageal ADC carcinogenesis. Exposure to these compounds is possible through occupational factors, chlorinated tap water consumption or tobacco smoke. The latter, which is a weakly associated risk factor for ADC, contains methyl chloride\(^34\). However, our present data did not allow us to estimate exposure to halogenated compounds in our population.

Another hypothesis is that \( \text{GST} \) could participate in chronic inflammation through leukotriene synthesis\(^3,5\). In particular, leukotriene A\(_4\) to C4 (LTC\(_4\)) conversion requires \( \text{GST} \) activity. Inflammation is a major etiologic factor for ADC and leukotrienes have been found to be mediators implicated in this process\(^5\). Furthermore, leukotriene LTD\(_4\), which is biosynthesized from LTC\(_4\), was found to induce contraction of the oesophagus and lower esophageal sphincter in animal models\(^15-16\). This phenomenon is likely to be involved in gastro-esophageal reflux, which constitutes the strongest risk factor for ADC. However, though \( \text{GSTT1} \) is also expressed in esophageal mucosa\(^4\), it remains unclear whether this enzyme contributes to LTC\(_4\) synthesis in this tissue.

The association between susceptibility to cancer and \( \text{GSTT1} \) genotypes could be also explained by depletion in intracellular glutathione in the presence of \( \text{GSTT1} \) enzyme. In this case, cells would be more sensitive to radical species produced during the inflammatory process observed among adenocarcinoma patients.

In conclusion, our study shows a different pattern of susceptibility to SCC and ADC of esophagus in a European high risk population. Whereas a slight susceptibility to SCC could be conferred by \( \text{CYP1A1*1A/*2C and GSTM1*2/*2} \) genotypes, a high frequency of \( \text{GSTT1*1/*1} \) genotype was found among ADC. These results are consistent with the association of SCC with tobacco exposure, as other tobacco-related cancers such as lung cancer were found to be moderately linked to \( \text{CYP1A1*2C allele and GSTM1*2/*2} \) genotype. In ADC, our results suggest 3 distinct hypotheses. (1) The activation of exogenous procarcinogens, such as small halogenated compounds (to which ways of exposure remain to be identified), by \( \text{GSTT1} \). Unlike tobacco, the evaluation of exposure to small halogenated compounds remains difficult because of the wide distribution of these compounds. (2) The contribution of \( \text{GSTT1} \) to the inflammatory response of esophageal mucosa, which is known to be a strong risk factor for ADC, possibly by way of leukotriene synthesis. (3) Higher sensitivity to the inflammatory process associated with intracellular depletion of glutathione. A new study focusing on esophageal ADC with a larger recruitment would allow us to investigate these issues.

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