FAS and FASL variations in outcomes of tobacco- and alcohol-related head and neck squamous cell carcinoma patients

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Abstract
Radiotherapy and cisplatin lead to cell killing in head and neck squamous cell carcinoma patients, but adverse events and response to treatment are not the same in patients with similar clinicopathological aspects. The aim of this prospective study was to evaluate the roles of TP53 c.215G > C, FAS c.-671A > G, FAS c.-1378G > A, FASL c.-844C > T, CASP3 c.-1191A > G, and CASP3 c.-182-247G > T single nucleotide variants in toxicity, response rate, and survival of cisplatin chemoradiation-treated head and neck squamous cell carcinoma patients. Genomic DNA was analyzed by polymerase chain reaction for genotyping. Differences between groups of patients were analyzed by chi-square test or Fisher’s exact test, multiple logistic regression analysis, and Cox hazards model. One hundred nine patients with head and neck squamous cell carcinoma were enrolled in study. All patients were smokers and/or alcoholics. Patients with FAS c.-671GG genotype, FAS c.-671AG or GG genotype, and FASL c.-844CC genotype had 5.52 (95% confidence interval (CI): 1.42–21.43), 4.03 (95% CI: 1.51–10.79), and 5.77 (95% CI: 1.23–27.04) more chances of presenting chemoradiation-related anemia of grades 2–4, lymphopenia of grade 3 or 4, and ototoxicity of all grades, respectively, than those with the remaining genotypes. FAS c.-671GG genotype was also seen as an independent predictor of shorter event-free survival (hazard ratio (HR): 2.05; P = 0.007) and overall survival (HR: 1.83; P = 0.02) in our head and neck squamous cell carcinoma patients. These findings present, for the first time, preliminary evidence that inherited abnormalities in apoptosis pathway, related to FAS c.-671A > G and FASL c.-844C > T single nucleotide variants, can alter toxicity and survival of tobacco- and alcohol-related head and neck squamous cell carcinoma patients homogeneously treated with cisplatin chemoradiation.

Keywords
Cisplatin, FAS, FASL, head and neck squamous cell carcinoma, single nucleotide variants

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Introduction

Head and neck (HN) squamous cell carcinoma (SCC) accounts for only 5% of all new cancer cases reported worldwide, but more than half of patients present advanced locoregional disease at diagnosis and have 5 years overall survival (OS) rates lower than 50%. HNSCC has tobacco smoking and alcohol consumption or human papillomavirus type 16 (HPV16) infection as risk factors.

Standard treatment for patients with locally advanced HNSCC, whose surgical resection is not amenable, is radiotherapy (RT) with cisplatin (CDDP). Ionizing radiation (IR) leads to DNA single- and double-strand breaks promoting cancer cell death. The cytotoxic radiation (IR) leads to DNA single- and double-strand breaks promoting cancer cell death. The cytotoxic radiation (IR) leads to DNA single- and double-strand breaks promoting cancer cell death. The variability in adverse events and tumor response induced by RT and CDDP among patients receiving the same treatment can be attributed to genetic changes causing modifications in cellular phenotype. Functional studies revealed that variant alleles of TP53 c.215G > C was previously associated with toxicity, response and survival of HNSCC patients treated with a homogeneous protocol consisting of RT and CDDP chemotherapy.

Materials and methods

Study population

In this prospective study, we evaluated 109 patients with HNSCC diagnosed at the Clinical Oncology Service of the General Hospital of University of Campinas between June 2011 and February 2014. The study was conducted according to the Declaration of Helsinki and was approved by the local Ethics Committee (number 274/2011), where all subjects provided written informed consent.

Patients were classified as smokers or non-smokers and drinkers or abstainers. HNSCC was diagnosed and staged according to standard criteria.

The presence of HPV16 in tumor fragments embedded in paraffin was analyzed by immunohistochemical staining and in situ hybridization as previously described.

Patients were treated with chemoradiation as definitive treatment due to locoregional unresectable tumor, refusal of surgery related to expected functional or anatomical sequel, or an organ preservation protocol. The treatment consisted of 35 sessions of RT being 2 Gy per session and intravenous CDDP at dose of 80–100 mg/m² on days 1, 22, and 43 (100 mg/m² of CDDP was administered to patients with Karnofsky Performance Scale (KPS) 80%–100% and without concomitance, and patients with KPS 60%–70% and without concomitance or KPS higher than 70% with concomitance received CDDP at dose of 80 mg/m²). Patients who presented adverse side effects of grades 3 or 4 received CDDP at lower dose in further administrations or had suspension of CDDP. As antiemetic premedication, metoclopramide (10 mg, every 6 h for 3 days) after each CDDP infusion. Adherence to prescriptions was considered in study.

Vomiting was assessed after CDDP infusion and during the four following days. Cytopenias were evaluated by hematological exams performed after CDDP infusion. Nephrotoxicity and ototoxicity were analyzed.

The aim of this study was to evaluate the roles of TP53 c.215G > C, FAS c.-671A > G, FAS c.-1378G > A, FASL c.-844 C > T, CASP3 c.-1191A > G, and CASP3 c.-182-247G > T SNVs in toxicity, response rate, and survival of HNSCC patients treated with a homogeneous protocol consisting of RT and CDDP chemotherapy.
Cancer Institute (NCI) criteria version 4.0,34 and the worst toxicity grade during treatment was considered for analysis.

The response to treatment was quantified by Response Evaluation Criteria in Solid Tumors (RECIST) 1.1.35 Patients who failed to respond to their initial treatment regimen or relapsed received intravenous methotrexate as palliative chemotherapy.36 Patients were followed at 3-month intervals and the latest follow-up was recorded in December 2018.

Genetic polymorphism analysis

*TP53* c.215G > C (rs1042522),37 *FAS* c.-671A > G (rs1800682),38 *FAS* c.-1378G > A (rs2234767) and *FASL* c.-844C > T (rs763110),39 *CASP3* c.-1191A > G (rs12108497),15 and *CASP3* c.-182-247G > T (rs4647601)40 genotypes were identified in genomic DNA by polymerase chain reaction and enzymatic digestion. Briefly, the regions of interest for each gene were amplified using the mix of sterile water, 10× buffer with (NH4)2SO4 (Thermo Scientific®, USA), MgCl2 (Thermo Scientific®, USA), 10 pmoles/µL of each primer (Integrated DNA Technologies®, USA), 2.5 U Taq DNA polymerase (Thermo Scientific®, USA), 25 mM MgCl2 (Thermo Scientific®, USA), 40 mM dNTP (Thermo Scientific®, USA), 10 pmol/µL of each primer (Integrated DNA Technologies®, USA), 2.5 U Taq DNA polymerase (Thermo Scientific®, USA), and 200 ng DNA. The amplification products were subjected to fragmentation with 5 U of each enzyme: BstUl (rs1042522 and rs2234767) (Thermo Scientific®, USA), MvaI (rs1800682) (Thermo Scientific®, USA), BsrDI (rs763110) (Thermo Scientific®, USA), Stul (rs12108497) (Thermo Scientific®, USA), and Hpy ch4V (rs4647601) (New England Biolabs®, USA).

Subsequently, the fragments were visualized on agarose gel stained with ethidium bromide. Positive and negative controls were used in reactions. The amount of PCR products was measured by densitometric analysis using the Researcher's Toolkit.41

The statistical power of analysis (PA) was calculated using univariate Cox proportional hazard ratio (HR) regression. All variables with *P*-value ≤ 0.15 were included in multivariate Cox regression analysis. Significant results were internally validated conducting on the final model via the bootstrap method using 1000 replications.

Significance results were two-sided and achieved when *P*-value was < 0.05. All tests were done using the SPSS 24.0 software (SPSS Incorporation, IL, USA).

Results

The patients’ median age was 56 years old. Most patients were males, smokers, and drinkers; the three non-smoker patients referred accentuated alcohol consumption and the nine non-drinker patients were important smokers. About 60% of patients had tumor in oral cavity or oropharynx and more than 80% of patients showed well or moderately differentiated tumor and tumors of III or IV stage. All analyzed cases tested negative for HPV16. Vomiting was reported in about 60% of cases, anemia was identified in about 97% of cases, leukopenia in 72% of cases, neutropenia in 60% of cases, lymphopenia in 97% of cases, and almost 30% of patients presented thrombocytopenia during or after treatment. About one-quarter of the available patients obtained a complete response with chemoradiation, while the remainder presented partial or stable disease (Table 1).

All patients received the total dose of RT (70 Gy). Eighty-five patients received three CDDP infusions and 24 patients were treated with two CDDP infusions due to the occurrence of myelotoxicity and/or nephrotoxicity. Median cumulative CDDP amount administered to patients was 260 mg/m² (range: 100–300 mg/m²). Medium or high adherence (97.7%) to antiemetics was observed among patients.

The median follow-up time of the 109 patients was 22 months (range: 3–89). The estimated probabilities of 24-month EFS and OS were 41.7% and 47.2%, respectively. At the study end, 34 patients were alive (6 of them were with HNSCC and 28 without HNSCC) and 75 patients died (67 of them by disease and 8 by others causes).

Association between genetic variants, toxicity, and response to therapy

When genotypes of SNVs were analyzed in patients stratified by bone marrow toxicity to chemoradiation, it was found an excess of *FAS* c.-671GG genotype in...
Table 1. Clinical and tumor aspects of 109 patients with head and neck squamous cell carcinoma.

| Variable                         | Median (range) or N (%) |
|----------------------------------|-------------------------|
| Age (years)                      | 56 (27–74)              |
| Gender                           |                         |
| Male                             | 101 (92.7)              |
| Female                           | 8 (7.3)                 |
| Tobacco consumption              |                         |
| Smokers                          | 106 (97.2)              |
| Non-smokers                      | 3 (2.8)                 |
| Alcohol consumption              |                         |
| Drinkers                         | 100 (91.7)              |
| Abstainers                       | 9 (8.3)                 |
| Tumor location                   |                         |
| Oral cavity or oropharynx        | 64 (58.7)               |
| Hypopharynx or larynx            | 45 (41.3)               |
| Histological grade\(^a\)         |                         |
| Well or moderately               | 73 (82.0)               |
| Poorly or undifferentated        | 16 (18.0)               |
| Tumor stage                      |                         |
| I or II                          | 6 (5.5)                 |
| III or IV                        | 103 (94.5)              |
| Human papillomavirus type 16\(^b\) |                     |
| Positive                         | 0 (0.0)                 |
| Negative                         | 57 (100.0)              |
| Toxicity\(^a,\(^b\)\)           |                         |
| Vomiting                         | 50 (56.8)               |
| Anemia                           | 95 (96.9)               |
| Leukopenia                       | 70 (71.4)               |
| Neutropenia                      | 58 (59.2)               |
| Lymphopenia                      | 95 (96.9)               |
| Thrombocytopenia                 | 36 (36.7)               |
| Ototoxicity                      | 68 (76.4)               |
| Nephrotoxicity                   | 73 (85.9)               |
| Response rate\(^c\)              |                         |
| Complete                         | 21 (23.9)               |
| Partial                          | 62 (70.4)               |
| Stable disease                   | 5 (5.7)                 |

N: number of patients.

\(^a\) The number of patients differed from the total quoted in the study (n = 109) because it was not possible to obtain consistent information in some cases.

\(^b\) Grades 1–4 for myelotoxicity and ototoxicity and 1–5 for nephrotoxicity.

patients with anemia grades 2–4 than in those without anemia or with anemia grade 1 (30.4% versus 14.3%, \(P = 0.01\); PA: 45.8%) after Bonferroni correction (Figure 1(a)); patients with \(\text{FAS} \cdot \text{c.-671GG}\) genotype had 5.52 more chance of presenting anemia grades 2–4 than those with \(\text{FAS} \cdot \text{c.-671AA}\) or AG genotype. \(\text{FAS} \cdot \text{c.-671AG}\) or GG genotype was also more common in patients with lymphopenia grade 3 or 4 than in those without lymphopenia or with lymphopenia grade 1 or 2 (80.0% versus 56.3%; \(P = 0.005\); PA: 71.9%) after Bonferroni correction (Figure 1(b)); patients with \(\text{FAS} \cdot \text{c.-671AG}\) or GG genotype had 4.03 more chance of presenting lymphopenia grade 3 or 4 than those with \(\text{FAS} \cdot \text{c.-671AA}\) genotype (Table 2).

The SNVs analyzed in this study did not alter the occurrence of vomiting, response to CDDP chemoradiation, and nephrotoxicity (Supplementary Table 1). However, an excess of \(\text{FASL} \cdot \text{c.-844CC}\) genotype was found in patients with ototoxicity (all grades) than in those without ototoxicity (38.2% versus 9.5%, \(P = 0.02\); PA = 75.4%) (Figure 1(c)); patients with \(\text{FASL} \cdot \text{c.-844CC}\) genotype had 5.77 more chance of presenting ototoxicity after therapy than those with \(\text{FASL} \cdot \text{c.-844CT}\) or TT genotype.

Association between genetic variants and survival

At 24 months of follow-up, lower EFS and OS were observed in patients with stage III or IV tumor (38.8% versus 83.3%; \(P = 0.02\)) and (44.1% versus 83.3%; \(P = 0.01\)), respectively, compared with those with tumor at stage I or II (Kaplan–Meier estimates). The variable mentioned above remained predictors of shorter EFS and OS in HNSCC patients in univariate analysis. Multivariate analysis showed that tumor at III or IV stage (HR: 9.77, \(P = 0.02\); \(P\text{bootstrap} = 0.02\)) and (HR: 9.74, \(P = 0.02\); \(P\text{bootstrap} = 0.01\)), and \(\text{FAS} \cdot \text{c.-671GG}\) genotype (HR: 2.05, \(P = 0.007\), \(P\text{bootstrap} = 0.02\)) and (HR: 1.83, \(P = 0.02\); \(P\text{bootstrap} = 0.045\)) were independent predictors of shorter EFS and OS, respectively (Table 3, Figure 1(d) and (e)), EFS and OS were not altered by \(\text{TTP53} \cdot \text{c.215G} > \text{C}\), \(\text{FASL} \cdot \text{c.-1378G} > \text{A}\) (Figure 1(f) and (g)), \(\text{FASL} \cdot \text{c.-844C} > \text{T}\) (Figure 1(h) and (i)), \(\text{CASP3} \cdot \text{c.-1191A} > \text{G}\), and \(\text{CASP3} \cdot \text{c.-182-247G} > \text{T}\) SNVs.

Discussion

We investigated, in this prospective study, the roles of \(\text{TTP53} \cdot \text{c.215G} > \text{C}\), \(\text{FAS} \cdot \text{c.-671AA} > \text{G}\) and \(\text{c.1378G} > \text{A}\), \(\text{FASL} \cdot \text{c.-844C} > \text{T}\), \(\text{CASP3} \cdot \text{c.-1191A} > \text{G}\) and \(\text{c.-182-247G} > \text{T}\) SNVs, and \(\text{FASL} \cdot \text{c.-844CC}\) genotype and \(\text{FASL} \cdot \text{c.-844AG}\) or \(\text{GG}\) genotype had 5.52 and 4.30 more chances of presenting anemia grades 2–4 and lymphopenia grade 3 or 4 with CDDP.

We initially observed that clinicopathological aspects of our patients were in general like those seen in patients from other parts of the world,\(^29,42–45\) which indicate that our sample was representative of HNSCC patients in medical practice settings. The more consistent difference between our cases and others seemed to occur in HNSCC carcinogenesis. Although tobacco and alcohol consumption and HPV16 infection were significant risk factors for HNSCC worldwide,\(^4\) HPV was not identified in our patients and was uncommon in those from other regions of the country.\(^45,46\)
Figure 1. Bars of genotypes frequencies in (a) patients without anemia or with anemia grade 1 (gray bars) versus patients with anemia grades 2–4 (black bars), (b) patients without lymphopenia or with lymphopenia grade 1 or 2 (gray bars) versus patients with lymphopenia grade 3 or 4 (black bars), and (c) patients without ototoxicity (gray bars) versus patients with ototoxicity grades (1–4) (black bars). *FAS c.-671GG genotype was more common in patients with anemia grades 2–4 than in those without anemia or with anemia grade 1 (30.4% versus 14.3%, \( P = 0.01 \); PA: 45.8%), FAS c.-671AG or GG genotype was more common in patients with lymphopenia grade 3 or 4 than in those without lymphopenia or with lymphopenia grade 1 or 2 (80.0% versus 56.3%; \( P = 0.005 \); PA: 71.9%), and FASL c.-844CC genotype was more common in patients with ototoxicity (38.2% versus 9.5%, \( P = 0.02 \); power of analysis: 75.4%); patients with the respective genotypes had 5.52 more chance of presenting anemia grades 2–4, 4.03 more chance of presenting lymphopenia grade 3 or 4, and 5.77 more chance of presenting ototoxicity compared with patients with other genotypes. Multivariate Cox regression curve for (d) event-free and (e) overall survival in patients with FAS c.-671AA or AG genotype (N = 83) versus GG genotype (N = 26), (f) event-free and (g) overall survival in patients with FAS c.-1378 GG or GA genotype (N = 102) versus AA genotype (N = 7), and (h) event-free and (i) overall survival in patients with FASL c.-844CC or CT genotype (N = 82) versus TT genotype (N = 27), adjusted by gender and tumor stage.
| Variable | Anemia | Leukopenia | Neutropenia | Lymphopenia | Thrombocytopenia |
|----------|--------|------------|-------------|-------------|-----------------|
|          | G0 + G1 | G2–G4      | G0–G2       | G3 + G4     | G0–G2          |
|          | N (%)   | N (%)      | N (%)       | N (%)       | N (%)           |
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(continued)
| Variable       | Anemia | Leukopenia | Neutropenia | Lymphopenia | Thrombocytopenia |
|----------------|--------|------------|-------------|-------------|-----------------|
|                | G0+G1 | G2-G4      | G0-G2       | G3+G4       | G0-G2           | G3+G4 | G0       | G1-G4   |               |
|                | N (%) | N (%)      | N (%)       | N (%)       | N (%)           | N (%)  | N (%)    | N (%)    |               |
| TT             | 5 (11.9) | 19 (33.9) | 23 (26.7) | 1 (8.3) | 23 (28.4) | 1 (5.9) | 11 (22.9) | 13 (26.0) | 18 (29.0) | 6 (16.7) |
| P-value        | 0.020 | 0.24 (0.03–2.03) | 5.78 (0.71–47.09) | 1.08 (0.41–2.81) | 0.48 (0.17–1.37) |
| OR (95% CI)    | 4.11 (1.24–13.57) | 0.24 (0.03–2.03) | 5.78 (0.71–47.09) | 1.08 (0.41–2.81) | 0.48 (0.17–1.37) |
| CASP3 c.-191A > G |        |            |            |            |                 |       |          |          |               |
| AA             | 15 (35.7) | 25 (44.6) | 36 (41.9) | 4 (33.3) | 32 (39.5) | 8 (47.1) | 17 (35.4) | 23 (46.0) | 30 (48.4) | 10 (27.8) |
| P-value        | 0.410 | 1.44 (0.40–5.15) | 0.73 (0.25–2.10) | 0.75 (0.32–1.75) | 2.43 (1.00–5.89) |
| OR (95% CI)    | 0.69 (0.28–1.66) | 0.24 (0.03–2.03) | 5.78 (0.71–47.09) | 1.08 (0.41–2.81) | 0.48 (0.17–1.37) |
| AG or GG       | 7 (46.3) | 31 (55.4) | 50 (58.1) | 8 (66.7) | 49 (60.5) | 9 (52.9) | 31 (64.6) | 27 (54.0) | 32 (51.6) | 26 (72.2) |
| P-value        | 0.575 | 0.565 | 0.565 | 0.519 | 0.48 |
| OR (95% CI)    | 0.24 (0.03–2.03) | 0.24 (0.03–2.03) | 0.24 (0.03–2.03) | 0.24 (0.03–2.03) | 0.24 (0.03–2.03) |
| AA or AG       | 35 (83.3) | 53 (94.6) | 77 (89.5) | 11 (91.7) | 72 (88.9) | 16 (94.1) | 43 (90.6) | 45 (90.0) | 56 (90.3) | 32 (88.9) |
| GG             | 7 (16.7) | 3 (5.4) | 9 (10.5) | 1 (8.3) | 9 (11.1) | 1 (5.9) | 5 (10.4) | 5 (10.0) | 6 (9.7) | 4 (11.1) |
| P-value        | 0.820 | 0.525 | 0.820 | 0.881 | 0.821 |
| OR (95% CI)    | 0.14 (0.09–6.74) | 0.14 (0.09–6.74) | 0.14 (0.09–6.74) | 0.14 (0.09–6.74) | 0.14 (0.09–6.74) |
| CASP3 c.-182–247G>T | | | | | |
| GG             | 15 (35.7) | 18 (32.1) | 30 (34.9) | 3 (25.0) | 25 (30.9) | 8 (47.1) | 17 (35.4) | 16 (32.0) | 22 (35.5) | 11 (30.6) |
| GT or TT       | 27 (64.3) | 38 (67.9) | 56 (65.1) | 9 (75.0) | 56 (69.1) | 9 (52.9) | 31 (64.6) | 34 (68.0) | 40 (64.5) | 25 (69.4) |
| P-value        | 0.545 | 0.500 | 0.204 | 0.919 | 0.619 |
| OR (95% CI)    | 1.32 (0.53–3.29) | 1.60 (0.40–6.38) | 0.50 (0.17–1.45) | 1.04 (0.44–2.46) | 1.25 (0.51–3.01) |
| GG or GT       | 36 (85.7) | 49 (87.5) | 76 (88.4) | 9 (75.0) | 71 (87.7) | 14 (82.4) | 42 (87.5) | 43 (86.0) | 56 (90.3) | 29 (80.6) |
| TT             | 6 (14.3) | 7 (12.5) | 10 (11.6) | 3 (25.0) | 10 (12.3) | 3 (17.6) | 6 (12.5) | 7 (14.0) | 6 (9.7) | 7 (19.4) |
| P-value        | 0.568 | 0.213 | 0.560 | 0.997 | 0.177 |
| OR (95% CI)    | 0.70 (0.21–2.34) | 0.23 (0.08–10.94) | 0.32 (0.09–1.13) | 0.23 (0.08–10.94) | 0.32 (0.09–1.13) |
| CASP3 + CASP3  |        |            |            |            |                 |       |          |          |               |
| GT             | 14 (33.3) | 20 (35.7) | 28 (32.6) | 6 (50.0) | 29 (35.8) | 5 (29.4) | 17 (35.4) | 17 (34.0) | 17 (27.4) | 17 (47.2) |
| Other haplotypes | 28 (66.7) | 36 (64.3) | 58 (67.4) | 6 (50.0) | 52 (64.2) | 12 (70.6) | 31 (64.6) | 33 (66.0) | 45 (72.6) | 19 (52.8) |
| P-value        | 0.499 | 0.241 | 0.616 | 0.987 | 0.049 |
| OR (95% CI)    | 0.13 (0.05–3.51) | 2.07 (0.61–7.00) | 0.15 (0.02–1.25) | 1.00 (0.43–2.35) | 2.36 (1.00–5.59) |

G: grade of toxicity; N: number of patients; OR: odds ratio; CI: confidence interval; NE: not evaluated.

ORs were adjusted by gender to lymphopenia, and by age and tumor stage to anemia. The total number of patients differed from the total quoted in the study (N = 109) because it was not possible to obtain consistent information about toxicities in some cases.

*Significant even after Bonferroni correction for multiple comparisons (corrected P-value = 0.01).

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Table 3. Association of clinicopathological aspects and P53 c.215G > C, FAS c.-671A > G and c.-1378G > A, FASL c.-844 C > T, CASP3 c.-1191A > G and c.-182-247G > T genotypes and FAS and CASP3 haplotypes of 109 head and neck squamous cell carcinoma patients with event-free survival and overall survival.

| Variable                        | Event-free survival | Overall survival |
|---------------------------------|---------------------|-----------------|
|                                 | N events/N total   | Univariate      | Multivariate       | N events/N total   | Univariate      | Multivariate       |
|                                 | P-value | HR (95% CI) | P-value | HR (95% CI) | P-value | HR (95% CI) | P-value | HR (95% CI) |
| Age (years)                     |          |             |          |             |          |             |          |             |
| <56                             | 40/56    | 0.24        | 1.31    | (0.82–2.08) | NE       | 38/56    | 0.79        | 1.06 | (0.67–1.67) | NE |
| >56                             | 34/53    | Reference   |           |             | NE       | 37/53    | Reference   |           |             |
| Gender                          |          |             |          |             |          |             |          |             |
| Male                            | 66/101   | 0.10        | Reference|           | 0.06    | Reference   | 0.04    | Reference   |
| Female                          | 8/8      | Reference   | 1.85    | (0.88–3.87) | 2.01    | (0.96–4.22) | 1.10    | (0.44–2.74) |
| Ethnic origin                   |          |             |          |             |          |             |          |             |
| White                           | 64/97    | 0.60        | Reference|           | NE       | 67/97    | 0.73        | 1.13 | (0.54–3.37) | NE |
| Non-white                       | 10/12    | Reference   | 1.19    | (0.61–2.33) | 2.01    | (0.96–4.22) | 1.13    | (0.54–3.37) |
| Tobacco consumption             |          |             |          |             |          |             |          |             |
| Smokers                         | 71/106   | 0.23        | 2.03    | (0.63–6.49) | NE       | 74/106   | 0.41        | 2.01 | (0.63–6.49) | NE |
| Nonsmokers                      | 3/3      | Reference   | 2.30    | (0.52–3.24) | NE       | 1/3      | Reference   |           |             |
| Alcohol consumption             |          |             |          |             |          |             |          |             |
| Drinkers                        | 69/100   | 0.57        | 1.30    | (0.52–3.24) | NE       | 70/100   | 0.39        | 1.48 | (0.59–3.68) | NE |
| Abstainers                      | 5/9      | Reference   |           |             | NE       | 5/9      | Reference   |           |             |
| Tumor location                  |          |             |          |             |          |             |          |             |
| Oral cavity or oropharynx       | 44/64    | 0.90        | Reference|           | NE       | 43/64    | 0.96        | 0.99 | (0.62–1.56) | NE |
| Hypopharynx or larynx           | 30/45    | Reference   | 1.03    | (0.64–1.64) | Reference| 1.03    | (0.64–1.64) | Reference |             |
| Histological grade              |          |             |          |             |          |             |          |             |
| Well or moderately              | 49/72    | 0.24        | Reference|           | NE       | 48/73    | 0.21        | 1.50 | (0.79–2.83) | NE |
| Poorly or undifferentiated      | 11/16    | Reference   | 1.47    | (0.76–2.84) | NE       | 12/16    | Reference   |           |             |
| Tumor stage                     |          |             |          |             |          |             |          |             |
| I or II                         | 1/6      | 0.04        | Reference| 7.28    | (1.00–52.58) | 0.02 | Reference   | 9.77 | (1.33–71.41) | 0.02 |
| III or IV                       | 73/103   | Reference   |           | Reference   | 7.40    | (1.10–57.46) | Reference | 9.74 | (1.33–70.92) |     |
| P53 c.215G > C                  |          |             |          |             |          |             |          |             |
| GG                              | 35/51    | 0.85        | Reference|           | NE       | 35/51    | 0.85        | Reference   |
| GC or CC                        | 39/58    | 0.95        | 0.60    | (1.51)     | NE       | 40/58    | Reference   | 1.04 | (0.66–1.64) |
| GG or GC                        | 66/98    | 0.89        | Reference| 0.95    | (0.45–1.98) | NE       | 68/98    | 0.73        | Reference   |
| CC                              | 8/11     | Reference   |           | Reference   | 7/11     | Reference   |           | Reference   |
| FAS c.-671A > G                 |          |             |          |             |          |             |          |             |
| AA                              | 23/34    | 0.77        | Reference|           | NE       | 22/34    | 0.31        | Reference   |
| AG or GG                        | 51/75    | 1.07        | 0.65    | (1.76)     | NE       | 53/75    | 1.29        | (0.78–2.12) |
| AA or AG                        | 54/83    | 0.07        | Reference| 1.59    | (0.95–2.67) | 0.007 | Reference   | 2.05 | (1.21–3.45) |
| GG                              | 20/26    | Reference   |           | Reference   | 19/26    | 1.52        | (0.90–2.56) | Reference   |
| FAS c.-1378G > A                |          |             |          |             |          |             |          |             |
| GG                              | 53/80    | 0.87        | Reference|           | NE       | 54/80    | 0.86        | Reference   |
| GA or AA                        | 21/29    | 0.96        | 0.57    | (1.59)     | NE       | 21/29    | 0.95        | (0.57–1.58) |     |

(continued)
| Variable | Event-free survival | Overall survival |
|----------|---------------------|-----------------|
|          | N events/N total    | Univariate      | Multivariate | N events/N total    | Univariate | Multivariate |
|          | P-value | HR (95% CI) | P-value | HR (95% CI) | P-value | HR (95% CI) | P-value | HR (95% CI) |
| GG or GA | 69/102 | 0.99 | Reference | NE | 71/102 | 0.54 | Reference | NE |
| AA       | 5/7 | 0.99 | (0.40–2.47) | NE | 4/7 | 0.73 | (0.26–2.01) | NE |
| FAS + FAS| 14/24 | 0.93 | (0.56–1.68) | NE | 17/24 | 0.90 | (0.56–1.66) | NE |
| GA       | 57/85 | 0.93 | Reference | NE | 58/85 | 0.90 | Reference | NE |
| Other haplotypes | 57/85 | 0.93 | Reference | NE | 58/85 | 0.90 | Reference | NE |
| FASL c.-844 C > T | 18/32 | 0.32 | Reference | NE | 21/32 | 0.96 | Reference | NE |
| CC       | 56/77 | 1.30 | (0.76–2.21) | NE | 54/77 | 1.01 | (0.61–1.67) | NE |
| CC or CT | 53/82 | 0.21 | Reference | NE | 56/82 | 0.48 | Reference | NE |
| TT       | 21/27 | 1.38 | (0.83–2.29) | NE | 19/27 | 1.20 | (0.71–2.03) | NE |
| CASP3 c.-1191 A > G | 33/45 | 0.39 | Reference | NE | 33/45 | 0.23 | Reference | NE |
| AA       | 41/64 | 0.82 | (0.51–1.29) | NE | 42/64 | 0.75 | (0.47–1.19) | NE |
| AG or GG | 68/98 | 0.29 | Reference | NE | 67/98 | 0.85 | Reference | NE |
| AA or AG | 6/11 | 0.63 | (0.27–1.47) | NE | 8/11 | 0.93 | (0.44–1.94) | NE |
| CASP3 c.-182–247 G > T | 24/39 | 0.59 | Reference | NE | 29/39 | 0.78 | Reference | NE |
| GG       | 50/70 | 1.14 | (0.70–1.85) | NE | 46/70 | 0.93 | (0.58–1.49) | NE |
| GT or TT | 65/96 | 0.86 | Reference | NE | 67/96 | 0.66 | Reference | NE |
| TT       | 9/13 | 0.94 | (0.46–1.89) | NE | 8/13 | 0.85 | (0.40–1.77) | NE |
| CASP3 + CASP3 | 26/36 | 0.45 | (0.74–1.93) | NE | 24/36 | 0.93 | (0.62–1.65) | NE |
| GT       | 48/73 | 1.19 | (0.74–1.93) | NE | 51/73 | 1.01 | (0.62–1.65) | NE |
| Other haplotypes | 48/73 | 1.19 | (0.74–1.93) | NE | 51/73 | 1.01 | (0.62–1.65) | NE |

N: number, HR: hazard ratio, CI: confidence interval, NE: not evaluated.
P-values ≤ 0.15 are presented in bold letters for univariate Cox analysis.

In multivariate Cox analysis adjusted by gender and tumor stage: \( P_{ bootstrap} = 0.02 \); \( P_{ bootstrap} = 0.02 \); \( P_{ bootstrap} = 0.01 \); \( P_{ bootstrap} = 0.04 \). The total number of patients differed from the total quoted in the study (N = 109) because it was not possible to obtain consistent information about histological grade in some cases.
chemoradiation than those with the remaining genotypes, respectively. Myelotoxicty was not altered by TP53 c.215G>C, FAS c.1378G>A, FASL c.-844C>T, CASP3 c.-1191A>G and c.-182-247G>T SNVs. Khrunin et al.\textsuperscript{17} found association of TP53 c.215CC genotype with severe neutropenia in ovarian cancer patients treated with CDDP-cyclophosphamide. The difference in results obtained in our study and Khrunin's study may be attributed to distinct types of tumors and treatments. To the best of our knowledge, the role of FAS c.-671A>G SNV in cytopenias induced by CDDP chemoradiation was not previously analyzed.

It is already known that CDDP may induce a deep transient erythropoiesis alteration leading to anemia.\textsuperscript{47,48} CDDP inhibits bone marrow cell proliferation by DNA damage,\textsuperscript{49} possibly due to apoptosis induced by FAS activation.\textsuperscript{9} Because the allele G of FAS c.-671A>G was previously associated with lower apoptosis of damaged cells,\textsuperscript{13} these results were not expected by us. However, activation of FAS/FASL was described as an important mechanism in erythroid maturation\textsuperscript{50} and regulation of red blood cell homeostasis.\textsuperscript{51} and Rehman et al.\textsuperscript{52} observed that G allele of FAS c.-671A>G increased aplastic anemia risk. Moreover, deletion of FAS in lymphocytes caused lymphopenia in mice,\textsuperscript{53} suggesting that FAS expression in lymphocytes is required for their survival and/or proliferation.\textsuperscript{54} So, we hypothesize that abnormalities in erythroid lymphocytes is required for their survival and/or proliferation. maturation and lymphocytes survival and/or proliferation transient erythropoiesis alteration leading to anemia by generating reactive oxygen species (ROS),\textsuperscript{55} and ROS can promote programmed cell death from activation of the FAS/FASL pathway in auditory hair cell.\textsuperscript{56}

Third, no association of TP53 c.215G>C, FAS c.-671A>G and c.1378G>A, FASL c.-844C>T, CASP3 c.-1191A>G and c.-182-247G>T with response to treatment was seen in this study. Shiraishi et al.\textsuperscript{20} analyzed association of TP53 c.215G>C SNV with response to platinum plus vinorelbine, paclitaxel, docetaxel, or gemcitabine in lung cancer patients, and observed that patients with the CC genotype obtained a better response than others. Again, the distinct types of tumor and treatments may explain differences of results found in both studies.

Fourth, we observed that HNSCC patients with FAS c.-671GG genotype had lower EFS and OS, respectively, than those with the remaining genotypes, but TP53 c.215G>C, FAS c.1378G>A, FASL c.-844C>T, CASP3 c.-1191A>G and c.-182-247G>T SNVs did not alter patients’ survival. Zhang et al.\textsuperscript{22} evidenced associations of FAS c.-671AG or GG and FASL c.-844CT or TT genotypes with lower DFS in SCCO patients treated with RT, chemotherapy, and/or surgery, and the associations were particularly seen in patients with HPV16-positive tumors. No significant associations between FAS c.1378G>A SNP and patients’ survival were found in their study. Shiraishi et al.\textsuperscript{20} found longer DFS and OS in lung cancer patients with CC genotype of TP53 c.215G>C treated with platinum plus vinorelbine, paclitaxel, docetaxel, or gemcitabine, and Azad et al.\textsuperscript{21} observed lower DFS in early-stage HNSCC patients radiation-treated with the CC genotype of TP53 c.215G>C compared with those with the remaining genotypes. FAS c.1378AA genotype was associated with higher OS in chemoradiation-treated HPV16-positive SCCO patients analyzed by Feng et al.\textsuperscript{23} Again, the opposed results obtained in our study and previously reported studies may be attributed to the different types of tumors, carcinogenesis, or treatments.

Since the FAS c.-671GG genotype was related to lower apoptosis of cancer cells,\textsuperscript{13} the association of genotype with lower EFS and OS in HNSCC patients is also biologically plausible, and probably results from tumor cells survival and proliferation.

At this time, it is important to comment that although Zhang et al.\textsuperscript{22} had seen association of FAS c.-671AG or GG genotype with lower DFS particularly in HPV16-positive SCCO patients, we found associations of the GG genotype with DFS and OS of HNSCC, which were strongly related to tobacco smoking and alcohol consumption.

Finally, we postulate that although we have not evaluated combined genotypes of distinct genes, we cannot exclude co-participation of TP53 c.215G>C, CASP3 c.-1191A>G, and CASP3 c.-182-247G>T SNVs in associations between FAS and myelotoxicty and patients’ survival and FASL and ototoxicity found in our study.

We are aware that our study has some inherent limitations, such as the relatively small number of patients and the lack of investigation of HPV16 in all tumor samples, which may have limited the obtaining and interpretation of the findings.

In conclusion, our data present, for the first time, associations of FAS c.-671A>G and FASL c.-844C>T with hematological toxicity, ototoxicity, and survival of tobacco- and alcohol-related HNSCC patients homogeneously treated with CDDP chemoradiation. Nevertheless, large studies with evaluation of
HPV-16 in tumor fragments are needed to validate our results and further explore the molecular mechanisms that underlie the observed associations, which may have future utility as clinical prognostic biomarkers.

**Declaration of conflicting interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by State of Sa˜o Paulo Research Foundation (FAPESP) (grant numbers 2012/01807-2, 2012/01418-6).

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**Supplemental material**

Supplemental material for this article is available online.

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