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Q21 The impact of *GGH -401C > T* polymorphism on cisplatin-based chemoradiotherapy
2 response and survival in cervical cancer

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ABSTRACT

Aims: Cervical cancer is the third most frequent cancer in women worldwide, mostly treated with 23
cisplatin-based chemoradiotherapy. Since it is known that folate metabolism might interfere with cisplatin 24
effectiveness, we intended to study the influence of the *Gamma Glutamyl Hydrolase -401C > T* polymorphism 25
in treatment response in cervical cancer. 26

Methods: We retrospectively reviewed the clinical data of 167 patients with bulky cervical cancer submitted 27
to cisplatin-based chemoradiotherapy. The genotypes of *GGH -401C > T* SNP were determined by real-time 28
PCR and statistical analysis was performed by χ^2 test and survival analysis. 29

Results: The genotypes of *GGH -401C > T* were significantly associated with the response to platinum-based 30
chemoradiotherapy. Treatment response was higher in patients carrying the CC genotype, who presented a 31
significant increased chance of treatment response (survival time in months/genotype: 91 for CC Vs 72 for 32
CT/TT; $p=0.035$, log rank test). A Cox regression analysis accordingly showed that the presence of the T al- 33
lele was significantly linked to a worse treatment response (HR = 3.036; CI 95% 1.032-8.934, $p=0.044$). 34

Conclusions: The results of our study suggested the potential interest of *GGH -401C > T* as a predictive factor of 35
the outcome of cervical carcinoma treated with cisplatin-based chemoradiotherapy. 36

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1. Introduction

32 Cervical carcinoma was the third most common cancer in women
33 in 2008. Since it is a platinum-sensitive disease, cisplatin-based
34 chemoradiotherapy is the standard of care for advanced cervical
35 cancer stages (IB2-IVA FIGO stages) (Candelaria et al., 2006; Ferlay
36 et al., 2010; Tewari and Monk, 2010). Although weekly cisplatin at
37 40 mg/m² for six weeks is the standard of care for locally advanced
38 cervical carcinoma in many cancer centers as ours, its optimal scheduling
39 and dosing have yet to be established due to the frequent development
40 of therapy resistance (Candelaria et al., 2006). Different mechanisms

41 have been proposed to reduce cisplatin response, including altered
42 drug accumulation, enhanced drug detoxification and DNA repair, or
43 upregulation of specific biochemical pathways (Ottone et al., 1997;
44 Siddik, 2003). Thus, the identification of molecular predictors of response
45 urges (Siddik, 2003). As patients' genetic background might change the
46 response, metabolism and toxicity of cytotoxic agents as cisplatin (Le et
47 al., 2005; Siddik, 2003), polymorphisms of genes coding enzymes in-
48 volved in drug or cell metabolism as well as the DNA synthesis and repair
49 have been studied (Kim et al., 2008). 60

61 Due to the synergism between alkylating radiosensitive agents
62 and radiotherapy, it is possible to get good local and systemic control
63 rates. However, what is the impact of molecular modulators on each
64 treatment modality on cervical cancer is still controversial. 64

65 GGH is a lysosomal enzyme that regulates intracellular folate
66 pools and folate metabolism homeostasis (Odin et al., 2003; 66
Organista-Navaa et al., 2010; Schneider and Ryan, 2006; Yin et
67 al., 2003). The *GGH -401C > T* SNP, which is one of its most com- 68
mon polymorphisms, is a promoter polymorphism that causes the loss 69
of an inhibitory transcription-factor binding-site. Due to its influence on 70
one-carbon metabolism and cell survival, its role in cervical carcinogen- 71
esis and treatment response is biologically plausible (Odin et al., 2003). 72

Abbreviations: GGH, *Gamma Glutamyl Hydrolase*; C, cytosine; T, thymine; SNP, single-nucleotide polymorphism; FIGO, International Federation of Gynecology and Obstetrics; Gy, Gray; RECIST, response evaluation criteria in solid tumors; PCR, polymerase chain reaction; A, Adenine; G, Guanine; SPSS, statistical packages for the social sciences; χ^2 , Chi-Square; OS, Overall survival; SD, standard deviation; HR, hazard ratio; CI, confidence interval; dTMP, thymidilate synthase.

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With this study, we intended to investigate the influence of the *GGH* – 401 C>T polymorphism in determining chemoradiotherapy response in cervical cancer.

2. Subjects and Methods

2.1. Subjects

We conducted an hospital-based retrospective study analyzing 167 Caucasian women from Northern Portugal with histologically confirmed cervical cancer IB2-IVA FIGO stages, admitted in the Portuguese Institute of Oncology, Porto, Portugal. All women were treated with first line cisplatin-based chemoradiotherapy in the Portuguese Institute of Oncology from Porto. Assessment of tumor stage was based on the FIGO system. All samples were obtained with the informed consent of the participants prior to their inclusion in the study, according to the declaration of Helsinki.

2.2. Concurrent chemoradiotherapy treatment

Chemotherapy regimen consisted of cisplatin (40 mg/m², iv) administered weekly in a total of six weeks. Concurrent radiotherapy consisted of pelvic external beam radiotherapy (for a total dose of 45–50 Gy of pelvic irradiation) and one to three intracavitary brachytherapy applications after the completion of external pelvic radiotherapy (cumulative dose at point A: 75 Gy; cumulative dose to point B: 55 Gy). For patients with lymph node metastasis, the treatment field was set to extend beyond the known extent of disease. From 167 patients evaluated for *GGH* –401C>T genotypes, 101 completed 6 cycles of the chemotherapy treatment. Hematological toxicity was the main factor causing treatment interruption.

2.3. Evaluation of chemotherapy response

Patients were followed on average for a period of 32 months. The response to cisplatin was estimated by the change in tumor size, which was measured by physical and CT exams performed before and after completing the prescribed treatment. The longest diameter of the lesion was measured. Using RECIST criteria, the response was graded as complete response (cervical lesion eradication), partial response (at least a 30% decrease in the longest diameter of the cervical lesion), stable disease (neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease) and progressive disease (at least a 20% increase in the longest diameter of the cervical lesion). Patients with complete or partial responses were classified as good responders and patients with stable or progressive disease were regarded as poor responders.

2.4. Sample collection and genotyping

Genomic DNA was extracted from blood samples by using QIAamp® DNA Blood Mini Kit (QIAGEN®). For the detection of the *GGH* –401C>T polymorphism, we used real-time polymerase chain reaction (PCR) by TaqMan allelic discrimination assay according to manufacture instructions. Probes used were flagged with VIC®/FAM™ dyes, respectively linked to the wild-type and the variant allele: CTGGCCAACCCAGGTCCTCGAGAGG[A/G]GAGGTTGGGTGCCCGGCC GAGTT. Results were analyzed on a sequence detection system ABI 7300, version 1.2.3 (Applied Biosystems, USA) Negative controls were included in each run and 10% of the samples' genotyping was repeated for quality control. Samples were tested in a blind fashion.

2.5. Statistical analyses

Analysis of data was performed using the computer software Statistical Package for Social Sciences (SPSS) for Windows (version 18.0).

Difference in frequencies of the *GGH* genotypes between different chemotherapy response groups were evaluated by χ^2 test, considering $p < 0.05$ statistically significant. Overall survival was defined as the time, in months, between diagnosis and either death or time of the last clinical evaluation. OS curves were plotted using the Kaplan–Meier method and were compared with the log-rank test. Multivariate analysis (Cox regression) was performed with variables considered important prognostic factors in cervical cancer, which were the tumor stage, histology, the presence of lymph node metastasis and the genotype *GGH* –401CC.

3. Results

3.1. Patient characteristics

The median age of patients at diagnosis was 47 years with a mean age of 48.58 years (SD = 12.78). The FIGO stages of the enrolled patients were as follow: 13 patients with IB2, 4 patients with IIA, 43 patients with IIB, 24 patients with III and IV stages. Most patients (80%) presented squamous cell carcinoma and no lymph node metastasis (94%). All patients received cisplatin-based chemoradiotherapy. From those, 71 patients carrying the CC genotype were good responders and 5 were poor responders. From patients carrying the CT/TT genotypes, 81 were good responders and 10 were poor responders (OR = 0.741, 95% IC 0.487–1.129; $p = 0.140$). Sample characteristics are reported in Table 1.

3.2. SNP genotyping

We analyzed *GGH* –401C>T polymorphism genotypes using the real-time PCR methodology. The genotype frequencies observed were 45.1% (78 cases) for CC genotype, 42.2% (73 cases) for CT heterozygous genotype and 12.7% (22 cases) for TT genotype. The genotypic frequencies were in genetic equilibrium according to the Hardy–Weinberg law

Table 1
Sample characterization.

| Variables | N | (%) | t.1.3 |
|--|-----|------|--------|
| Smoking habits | | | t.1.4 |
| Yes | 23 | 15.5 | t.1.5 |
| No | 115 | 77.7 | t.1.6 |
| Unknown | 10 | 6.8 | t.1.7 |
| Oral contraceptives | | | t.1.8 |
| Yes | 70 | 45.5 | t.1.9 |
| No | 70 | 45.5 | t.1.10 |
| Unknown | 14 | 9 | t.1.11 |
| Tumor FIGO stage | | | t.1.12 |
| IB2 | 13 | 7.6 | t.1.13 |
| IIA | 4 | 2.4 | t.1.14 |
| IIB | 43 | 32.6 | t.1.15 |
| IIIA | 2 | 1.2 | t.1.16 |
| IIIB | 20 | 11.6 | t.1.17 |
| IVA | 2 | 1.2 | t.1.18 |
| Histology | | | t.1.19 |
| Squamous cell carcinoma | 139 | 80.3 | t.1.20 |
| Adenocarcinoma | 22 | 12.7 | t.1.21 |
| Adenosquamous | 7 | 4.0 | t.1.22 |
| Others | 5 | 2.9 | t.1.23 |
| Lymph node metastasis | | | t.1.24 |
| Yes | 7 | 5.9 | t.1.25 |
| No | 112 | 94.1 | t.1.26 |
| Number of chemotherapy cycles completed | | | t.1.27 |
| 1–3 | 6 | 3.7 | t.1.28 |
| 4–6 | 151 | 83.5 | t.1.29 |
| Treatment response | | | t.1.30 |
| CR | 123 | 71.9 | t.1.31 |
| PR | 29 | 17.0 | t.1.32 |
| SD | 9 | 5.3 | t.1.33 |
| DP | 6 | 3.5 | t.1.34 |

CR: complete response; PR: partial response; SD: stable disease; DP: disease progression.

156 ($\chi^2 = 0.32$). Genotypic differences between cancer subtypes were addi- 195
 157 tionally tested but no statistically significant differences were observed 196
 158 (data not shown).

159 3.3. Response to chemotherapy in relation to *GGH* -401C>T polymorphism

160 Regarding mean survival time, we used the Kaplan–Meier methodol- 195
 161 ogy to calculate the differences between the survival times of women 196
 162 carrying the CC genotype and the CT/TT genotypes. A statistically signif- 197
 163 icant association between the *GGH* -401C>T polymorphism and overall 198
 164 survival was observed in the univariate analysis [survival time in 199
 165 months/genotype: 91 for CC (95% CI 82,77 - 99,33) Vs 72 for CT/TT 200
 166 (95% CI 60,80 - 82,68); $p = 0.035$, log rank test]. Accordingly, the multi- 201
 167 variate analysis (Cox regression) adjusted to tumor stage, histology and 202
 168 the presence of lymph node metastasis showed that *GGH* genotypes 203
 169 were significantly linked to treatment response. Accordingly, individuals 204
 170 carrying the T allele had 3 times more risk of death relatively to women 205
 171 carrying the CC genotype (HR = 3.036; CI 95% 1.032-8.934, $p = 0.044$) 206
 172 (Fig. 1).

173 4. Discussion

174 Although evidence suggests that folate deprivation acts synergistical- 213
 175 ly with alkylating agents (Courtemanche et al., 2004; Novakovic et al., 214
 176 2006; Whiteside et al., 2006), the activation of compensatory mech- 215
 177 anisms leading to cell survival have been described. According to 216
 178 (Hayashi et al., 2007), who studied folate depletion in colon cancer 217
 179 cells, folate deprivation and disrupted one-carbon metabolism could be 218
 180 compensated by adaptive mechanisms that enable cells to maintain crit- 219
 181 ical one-carbon metabolism reactions (Hayashi et al., 2007). The devel- 220
 182 opment of a survival advantage was also observed in Chinese Hamster 221
 183 Ovary cells resistant to the growth-limiting effects of folate depletion, 222
 184 enabling them to better withstand cisplatin cytotoxicity (Branda et al., 223
 185 1998). (Cole et al., 2001) also described compensatory changes suscepti- 224
 186 ble of affecting drug sensitivity after *GGH* overexpression in MCF7 cells 225
 187 (Cole et al., 2001).

188 Since the *GGH* -401C>T polymorphism leads to *GGH* overexpression, 226
 189 we thought that an impairment in folate metabolism might activate com- 227
 190 pensatory pathways involved in DNA repair and maintenance of cell sur- 228
 191 vival. According to our main hypothesis, the *GGH* -401C>T SNP might be 229
 192 involved in the development of cisplatin-based chemoradiotherapy res- 230
 193 istance. The fact cisplatin causes an increase in the intracellular levels 231
 194 of 5,10-methylene-tetrahydrofolate and tetrahydrofolate, and enhances 232

the gene expression of enzymes involved in dTMP synthase cycle sup- 195
 ports our suggestion (Lu et al., 1988; Scanlon and Kashani-Sabet, 1988; 196
 Whiteside et al., 2006).

According to our results, the CC carriers had a significantly 197
 higher overall survival than the T allele carriers [survival time in 198
 months/genotype: 91 for CC (95% CI 82,77 - 99,33) Vs 72 for CT/TT 199
 (95% CI 60,80 - 82,68); $p = 0.035$, log rank test]. A multivariate analysis 200
 supported this observation, showing that patients carrying the T allele 201
 had 3 times more risk of death relatively to women carrying the CC ge- 202
 notype (HR = 3.036; CI 95% 1.032-8.934, $p = 0.044$) (Fig. 1). However, 203
 it is important to note that folate depletion poses different metabolic 204
 stresses in cells depending on the cell type, which will result in different 205
 adaptive regulation of folate metabolism enzymes (Hayashi et al., 2007; 206
 Novakovic et al., 2006). A cDNA microarray analysis of a human squa- 207
 mous cell carcinoma cell line treated with cisplatin for 5 days accord- 208
 209 210 211 212

213 5. Conclusions

On the light of these results we might suggest that this polymor- 213
 phism might be a predictive factor of the outcome of cervical carcino- 214
 ma treated with cisplatin-based chemoradiotherapy, though future 215
 and larger studies would be necessary to confirm it. As *GGH* is a non- 216
 specific enzyme, whose expression is dependent on the analyzed tissue, 217
 it might not be appropriate to draw a generalized conclusion regarding 218
 other cancer models, whose folate requirements for growth are differ- 219
 ent. The fact we could not study other genes involved in folate metabo- 220
 lism, DNA repair or treatment response is one drawback of this study. 221

Although an increase in neoadjuvant cisplatin-based chemotherapy 222
 response for cervical cancer patients carrying the CC genotype has al- 223
 ready been observed by (Chung et al., 2006), to the best of our knowledge 224
 there are currently no published studies on the relationship between 225
GGH -401C>T polymorphism and cervical cancer chemoradiotherapy 226
 response. 227

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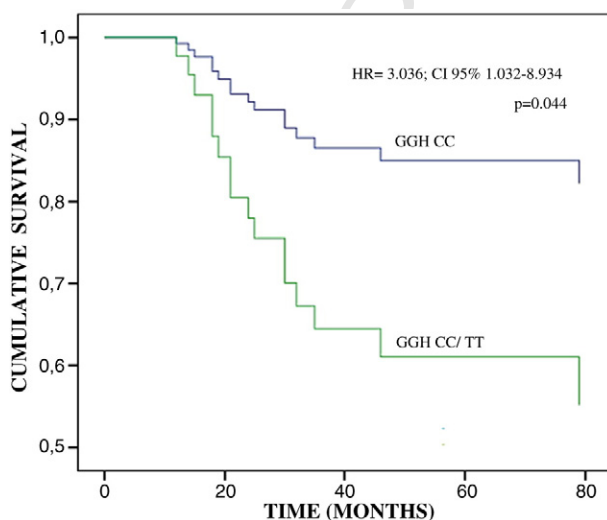


Fig. 1. Cox regression analysis of cervical cancer patients adjusted with FIGO stage, tumor histology and the presence of lymph node metastasis as covariates, according to *GGH* -401C>T genotypes (HR = 3.036; CI 95% 1.032-8.934, $p = 0.044$).

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