

Single nucleotide polymorphism of *GSTP1* and pathological complete response in locally advanced rectal cancer patients treated with neoadjuvant concomitant radiochemotherapy

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Purpose: Standard treatment for locally advanced rectal cancer consists of neoadjuvant radiochemotherapy with concomitant fluoropyrimidine or oxaliplatin and surgery with curative intent. Pathological complete response has shown to be predictive for better outcome and survival; nevertheless there are no biological or genetic factors predictive for response to treatment. We explored the correlation between the single nucleotide polymorphisms (SNPs) *GSTP1* (A313G) and *XRCC1* (G28152A), and the pathological complete response and survival after neoadjuvant radiochemotherapy in locally advanced rectal cancer patients.

Materials and Methods: Genotypes *GSTP1* (A313G) and *XRCC1* (G28152A) were determined by pyrosequencing technology in 80 patients affected by locally advanced rectal cancer.

Results: The overall rate of pathological complete response in our study population was 18.75%. Patients homozygous AA for *GSTP1* (A313G) presented a rate of pathological complete response of 26.6% as compared to 8.5% of the AG+GG population ($p = 0.04$). The heterozygous comparison (AA vs. AG) showed a significant difference in the rate of pathological complete response (26.6% vs. 6.8%; $p = 0.034$). *GSTP1* AA+AG patients presented a 5- and 8-year cancer-specific survival longer than *GSTP1* GG patients (87.7% and 83.3% vs. 44.4% and 44.4%, respectively) ($p = 0.014$). Overall survival showed only a trend toward significance in favor of the haplotypes *GSTP1* AA+AG. No significant correlations were found for *XRCC1* (G28152A).

Conclusion: Our results suggest that *GSTP1* (A313G) may predict a higher rate of pathological complete response after neoadjuvant radiochemotherapy and a better outcome, and should be considered in a more extensive analysis with the aim of personalization of radiation treatment.

Keywords: Rectal cancer, Radiochemotherapy, Single nucleotide polymorphism, Prognostic factor, Tumor response, Genetic marker

Introduction

Locally advanced rectal cancer represent 33% of newly

diagnosed rectal cancer with 5-year survival of 69.5% [1]. Treatment of choice is represented by neoadjuvant radiochemotherapy followed by surgery, that allow to perform

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a more conservative, sphincter saving, surgery, as compared to surgery alone [2-5].

Despite advances in neoadjuvant strategies local relapse occurs in 7%–10% of patients, especially in those who did not have a pathological complete response. Local relapse is in-field or marginal in 79% of the cases, with 3- and 5-year overall survivals of 36.7%, and 19.1%, respectively [6,7].

The pathological complete response has been proven to be correlated with a lower incidence of local recurrence and is predictive of longer survival [8]. Therefore, the identification of biological or genetic factors predictive to pathological complete response may help physicians in the personalization of the treatment. However, at the moment there are no proven factors routinely used in the clinical practice.

Radiotherapy applies its cytotoxic effects through the damage to cell structures, proteins, and DNA. Mechanisms of radiotherapy damage are essentially delivered indirectly, by reactive oxygen intermediate, and directly by damage to the cellular structures. Individual variations in the pathways of DNA repair and xenobiotic metabolism can be implicated in the mechanisms of radiosensitivity.

Recently, the correlation between genetic mutation and the development of side effects in rectal cancer patients has been investigated [9-13]. Mutations regarding genes involved in the regulation of cell cycle, apoptosis, and tumorigenesis, expression of growth factor receptors, nucleoside synthesis, and DNA repair has been considered and analyzed. The normal human genome presents numerous germinal variations in DNA sequences called polymorphisms. These variations are represented in more than 90% of cases by single-nucleotide polymorphisms (SNPs) [14], some of which seem to be associated with the response to neoadjuvant radiochemotherapy in rectal cancer [15,16].

The further examination of the genetic variation of the mechanisms of DNA repair may suggest in the future a potential benefit of intensified treatments in selected subgroups. Furthermore, inefficient treatments could be avoid in unfavourable candidates.

The aim of the study is to evaluate the role of the SNPs *GSTP1* (glutathione S-transferase pi) A313G (rs1695) and *XRCC1* (x-ray repair cross-complementing 1) G28152A (rs25487) as predictive markers of pathological complete response in locally advanced rectal cancer patients treated with neoadjuvant radiochemotherapy. Also overall survival and cancer-specific survival were assessed.

Materials and Methods

1. Patients

Between November 2008 and June 2015, 85 patients affected by locally advanced rectal cancer receiving neoadjuvant radiochemotherapy were included. At the final revision, complete data regarding preoperative and postoperative imaging, genetic assessment and histopathological evaluation were available for 80 patients that represent the study population. Patients' characteristics are summarized in Table 1.

Table 1. Patients' characteristics and clinical outcome (n = 80)

Characteristic	Value
Age (yr)	64(36–85)
Sex	
Male	49(61.25)
Female	31(38.75)
T stage	
T2	9(11.25)
T3	67(83.75)
T4	4(5.00)
N Stage	
N0	37(46.25)
N1	32(40.00)
N2	11(13.75)
Distance from anal verge (cm)	
≤5	44(55.00)
>5	36(45.00)
Surgery procedure	
≤5 cm	69(86.25)
RAR	10(12.50)
APR	1(1.25)
TAE	-
ypT stage	
T0	15(18.75)
T1	4(5.00)
T2	22(27.50)
T3	38(47.50)
T4	1(1.25)
ypN stage	
N0	60(75.00)
N1	16(20.00)
N2	4(5.00)
Dworak TRG sec.	
0	7(8.75)
1	17(21.25)
2	28(35.00)
3	13(16.25)
4	15(18.75)

Values are presented as median (range) or number (%).

AR, rectum anterior resection; APR, abdominoperineal resection; TAE, transanal excision; TRG, tumour regression score.

Inclusion criteria were: age ≥ 18 years, biopsy-proven diagnosis of adenocarcinoma localized ≤ 10 cm from the anal verge, locally advanced rectal cancer (stage II–III), complete staging including colonoscopy, contrast enhanced multi parametric-MRI of the pelvis and total body CT. Exclusion criteria were: evidence of distant metastases, inability to receive radiotherapy associated to chemotherapy.

2. Treatment

The radiation treatment was delivered using a three-dimensional conformal technique. The clinical target volume 1 (CTV1) included the entire macroscopic tumour, the mesorectum, internal iliac and presacral lymph nodes, adding a margin of 1 cm in all directions to generate the corresponding planning treatment volume 1 (PTV1). The boost volume (CTV2) included the macroscopic tumour and the corresponding mesorectum for 1–1.5 cm in craniocaudal direction from the external margin of the tumor. Subsequently, a margin of 1 cm in all directions was added to generate the PTV2. The total prescribed dose to the PTV1 was 45 Gy delivered in 25 fractions of 1.8 Gy per day for 5 days a week over 5 weeks, plus a concomitant boost of 10 Gy in 10 fractions delivered biweekly to the PTV2 (total dose, 55 Gy).

All patients received concurrent chemotherapy: 48 patients (60%) received capecitabine (825 mg/mq) twice daily and 32 patients (40%) received a daily continuous infusion of 5-fluorouracil (225 mg/mq/day) on each day of radiotherapy. Contrast enhanced multi parametric-MRI of the pelvis was performed for each patient to evaluate the tumour response after 4 weeks of neoadjuvant radiochemotherapy. A sphincter-saving surgery was evaluated taking into account the response to combined treatment and the distal from anal verge, and was performed 6–8 weeks after neoadjuvant radiochemotherapy. Sixty-nine patients (86.25%) received an anterior resection, 10 patients (12.5%) an abdominoperineal resection, and 1 patient (1.25%) a transanal endoscopic microsurgery. Adjuvant chemotherapy was administered in 25 patients (31.25%), 16

(64%) received FOLFOX, and 9 (36%) XELOX.

3. Gene selection and genotype analysis

After a careful review of the literature candidate SNPs *GSTP1* A313G (rs1695) and *XRCC1* G28152A (rs25487), were chosen on the basis of a radiation-related mechanism of action and previous studies that included large population or that were perspective [15,16]. Genes directly linked to the metabolism of fluoropyrimidines were not chosen to avoid possible confounding factors.

Blood sample was collected before the start of treatment and genomic DNA was isolated using the X-tractor Gene system (Corbett Life Science, Sydney, Australia). *GSTP1* A313G (rs1695) and *XRCC1* G28152A (rs25487) were examined. Reference sequences for the genes were obtained from NCBI GenBank database (<http://www.ncbi.nlm.nih.gov/>). The analyzed SNPs were chosen based on previous evidence and the high prevalence in white population. The Hardy-Weinberg equilibrium was fulfilled for the genotyped sites ($p > 0.1$, χ^2 test) and the prevalence of alleles had similar distribution compared with the Caucasian population, as shown in Table 2. Genotyping was performed by pyrosequencing technology, using the pyrosequencer (PyroMark ID system; Qiagen, Hilden, Germany) according to manufacturers' instructions. Both the amplification and the sequencing primers were obtained by the PSQ Assay Design software (Biotage AB and Biosystems, Uppsala, Sweden). Sequence of selected primers is reported in Table 3. The region covering the SNPs of interest was amplified by polymerase chain reaction (PCR). The PCR requirements were: 95°C for 3 minutes; 40 cycles with denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, and elongation at 72°C for 30 seconds; a final extension step at 72°C for 5 minutes. The PCRs were performed in a final volume of 25 μ L (except for *GSTP1*, for which 50 μ L were used), containing 70 ng of genomic DNA, 10 pmol of primers, 0.2 mM dNTPs, PCR buffer, 1 mM MgCl₂, and 1 U of Taq DNA polymerase (Takara Bio Inc., Otsu, Japan).

Table 2. Localization and observed frequency of the analyzed SNPs

SNP	Chromosomal localization ^{a)}	Amino acid position	Genotype ^{b)}	No. of patients	MAF expected (observed)
<i>GSTP1</i> A313G (rs1695)	11:67585218	Codon 105–313	A/G	80	0.33 (0.3)
<i>XRCC1</i> G28152A (rs25487)	19:43551574	Exon 10–399	G/A	80	0.34–0.36 (0.38)

SNP, single nucleotide polymorphisms; MAF, minor allele frequency.

^{a)}Chromosome indicates position. ^{b)}Genotype nomenclature refers to the DNA sense strand.

Table 3. Primers for PCR amplification and pyrosequencing

SNP	Forward primer (5'-3')	Reverse primer (5'-3')	Sequencing primer (5'-3')
<i>GSTP1</i> A313G (rs1695)	^a GTGGACATGGTGAATGAC	GCTCACATAGTTGGTGTAGA	GTTGGTGTAGATGAGGG
<i>XRCC1</i> G28152A (rs25487)	^a AGTACAGCCAGGTCCTAG	CGCTCCTCTCAGTAGTCT	CGTGTGAGGCCTACC

Primers are tagged with a biotin molecule attached.

PCR, polymerase chain reaction.

4. Follow-up and statistics

Pathologic response of the tumours was assessed by postoperative histopathological evaluation, according to the tumour regression grade (TRG) by Dworak Scale [17]. The tumour response in our series was therefore classified as follow: pathological complete response (TRG 4), no tumour cells, only fibrotic mass; major pathological response (TRG 3), very few tumour cells in fibrotic tissue with or without mucous substance; good pathological response (TRG 2), dominant fibrotic change with few poor tumour cells or groups; poor pathological response (TRG 1), dominant tumour mass with obvious fibrosis and/or vasculopathy; and absent pathological response (TRG 0), no regression.

Patients were classified into three groups on the basis of the allelic assessment (normal homozygous, mutated homozygous, heterozygous). Each group was then divided as non-complete responder (TRG 0-3) or complete responder (TRG 4). A univariate model using a χ^2 test (in 2 x 2 contingency tables) was performed in different genetic comparison models, including homozygote comparison (AA vs. aa), heterozygote comparison (Aa vs. aa; Aa vs. AA), dominant model (aa vs. Aa/AA), and recessive model (AA vs. Aa/AA). Overall survival and cancer-specific survival were estimated for patients with ≥ 12 months of available follow-up using the Kaplan-Meier method. Prognostic factors such as age, sex, clinical stage, pathological T and N, type of surgery, adjuvant chemotherapy, TRG and SNPs assessment were included in the univariate analysis using the log-rank test. Cox regression method and multiple logistic regression were used in multivariate analysis. Statistical analysis was performed using the SPSS statistical software package version 22.0 (IBM, Armonk, NY, USA). A $p \leq 0.05$ indicated a significant association.

Results

1. Treatment outcome and association to *GSTP1* (A313G)

Fifteen patients (18.75%) had a pathological complete response: 12 (80%) of them were homozygous AA, 2 (13.5%) were heterozygous AG, and 1 (6.5%) was homozygous GG.

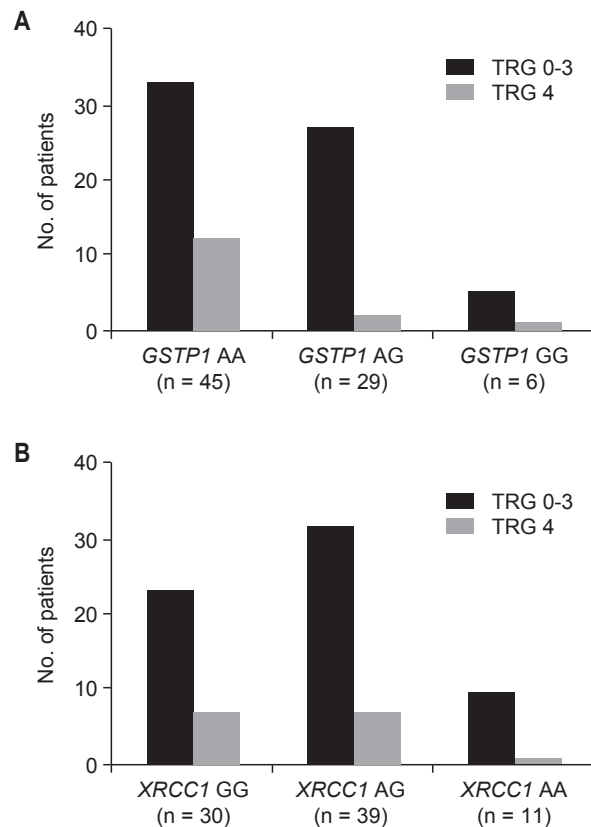


Fig. 1. Single nucleotide polymorphisms characteristics and response to treatment according to allelic assessment (n = 80). *GSTP1*, glutathione S-transferase pi; *XRCC1*, X-ray repair cross-complementing 1; TRG, tumour regression grade.

The distribution of the pathological response in our study population is reported in Fig. 1A.

Among the patients homozygous AA we observed a pathological complete response rate of 26.6% while in the population AG+GG the rate was 8.5% ($p = 0.04$). The heterozygote comparison (AA vs. AG) showed a significant difference in the rate of pathological complete response (26.6% vs. 6.8%; $p = 0.034$). No correlations have been found between *GSTP1* (A313G) and downstaging of primary tumor or lymph nodes.

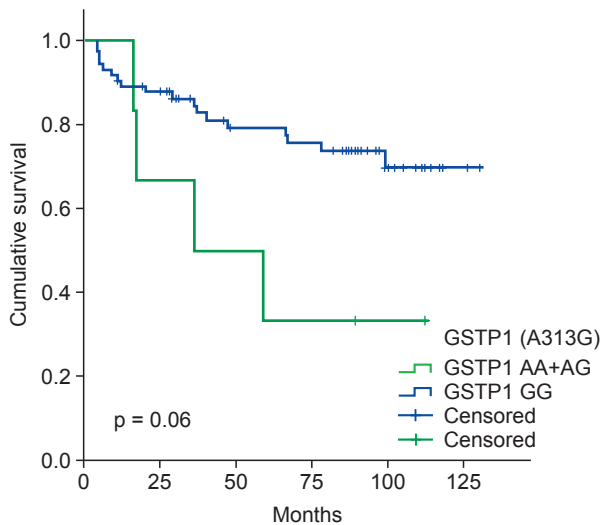


Fig. 2. Overall survival stratified for *GSTP1* AA+AG vs. GG.

The median overall survival was not reached; actuarial overall survival is 73.3%. The 5- and 8-year overall survivals stratified for AA vs. AG vs. GG were 72.9% and 61.1%, 76.9% and 76.9%, and 33.3% and 33.3%, respectively ($p = 0.075$). When compared GG vs. AA+AG the difference in overall survival showed a trend towards significance with rates at 5- and 8-year of 33.3% and 33.3%, 74.6% and 66.8%, respectively ($p = 0.06$) (Fig. 2).

Median cancer-specific survival was not reached; actuarial cancer-specific survival is 84%. The 5- and 8-year cancer-specific survivals stratified for AA vs. AG vs. GG were: 89.1% and 82%, 85.1% and 85.1%, 44.4% and 44.4%, respectively. A significant difference between groups was found ($p = 0.048$). The cancer-specific survivals between the haplotype GG and the combined population AA+AG at 5 and 8 years was 44.4% and 44.4%, 87.7% and 83.3%, respectively ($p = 0.014$) (Fig. 3).

2. Treatment outcome and association to *XRCC1* (G28152A)

The rate of pathological complete response for the haplotypes AA, AG, and GG was 9%, 17.9%, and 23.3%, respectively (Fig. 1B). This difference was not statistically significant in all the analyzed models.

The 5- and 8-year overall survivals for the haplotypes of *XRCC1* (G28152A) was as follows: GG (69.5% and 54.8%), AG (76.3% and 76.3%), and AA (45% and 45%) ($p = 0.145$). The 5- and 8-year cancer-specific survivals stratified for haplotypes were as follows: GG (82.7% and 77.2%), AG (90.7% and 90.7%), and AA (52.5% and 52.5%) ($p = 0.072$).

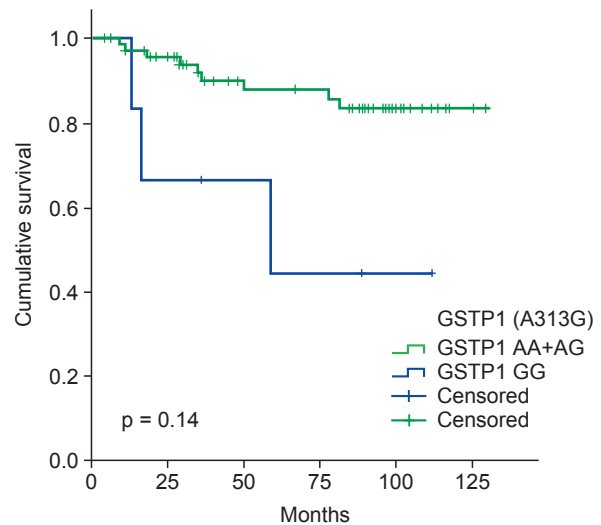


Fig. 3. Cancer-specific survival stratified for *GSTP1* AA+AG vs. GG.

No correlations have been found between *XRCC1* (G28152A) and down-staging of primary tumor and lymph nodes.

3. Pattern of relapse

After a median follow-up of 83 months (range, 4 to 130 months), local relapse occurred in 8 patients (10%), regional relapse occurred in 1 patient (1.25%; external iliac lymph nodes), and systemic relapse in 20 patients (25%). Seventy-five patients (93.75%) were evaluated for survival.

Systemic relapse occurred more frequently in patients homozygous GG for *GSTP1*, as compared to AA and AG (83.3%, 23.3%, and 21.4%, respectively; $p = 0.006$) and in patients with $\gamma N2$ nodal status as compared to $\gamma N1$ and $\gamma N0$ (66.7%, 46.7%, and 20.3%, respectively; $p = 0.036$). *GSTP1* GG was not correlated with higher rate of $\gamma N2$ nodal status.

4. Prognostic factors

At the univariate analysis, the presence of the gene G of *GSTP1* (A313G) correlated significantly with a lower overall survival ($p = 0.046$); moreover pathological nodal status (γN) ($p = 0.013$) and *GSTP1* GG ($p = 0.028$) were significantly correlated with a lower cancer-specific survival. At the multivariate analysis, *GSTP1* GG ($p = 0.039$; HR = 0.27; confidence interval, 0.12–0.31) was a significant factor associated with a lower cancer-specific survival, but not with overall survival. Moreover *GSTP1* GG significantly correlated with a lower incidence of pathological complete response ($p = 0.049$; OR = 0.2; confidence interval, 0.42–0.99) (Tables 4, 5).

Table 4. Multivariate analysis: overall survival vs. cancer-specific survival

Factor	Overall survival			Cancer-specific survival		
	HR	CI	p-value	HR	CI	p-value
Age (≥65 yr)	1.07	0.95–1.03	0.873	0.28	0.07–0.11	0.072
Sex	0.97	0.43–2.19	0.951	1.16	0.33–4.09	0.812
N down-staging	0.85	0.36–1.98	0.718	0.34	0.08–1.36	0.128
<i>GSTP1</i> GG	0.44	0.14–1.4	0.178	0.27	0.12–0.31	0.039
TRG 4	0.50	0.1–2.94	0.15	0.70	0.62–1.13	0.6

HR, hazard ratio; CI, confidence interval; *GSTP1*, glutathione S-transferase pi; TRG, tumour regression grade.

Table 5. Multivariate analysis of TRG 4

Factor	TRG 4		
	OR	CI	p-value
<i>GSTP1</i> AA	0.37	0.20–4.90	0.451
<i>GSTP1</i> GG	0.20	0.42–0.99	0.049

TRG, tumour regression grade; OR, odds ratio; CI, confidence interval; *GSTP1*, glutathione S-transferase pi.

Discussion and Conclusion

The gold standard for the treatment of locally advanced rectal cancer in Western countries is neoadjuvant radiochemotherapy followed by surgery with curative intent. Many studies have evaluated clinical, pathological and molecular factors to predict for the prognosis of rectal cancer after neoadjuvant radiochemotherapy. Nevertheless, the histopathological classification of the surgical specimen remains the main parameter to define patients' prognosis. Current available data are not still sufficient to modify standard therapies into individualized treatments. Intensified regimens of radiochemotherapy could lead to a substantial increase in side effects and may be considered only for patients with very advanced stage or at high risk of local/distant relapse. On the other hand, inadequate therapy may worsen the outcome and can be considered only in very early stage disease.

We hypothesized that modifications in the system of reactive oxygen intermediate detoxification and in the DNA repair mechanism may lead to an increased efficacy of radiotherapy that can be translated in an increased response to treatment. In the present study, the correlation between the SNPs *GSTP1* (A313G) and *XRCC1* (G28152A) and pathological response, survival and pattern of relapse in locally advanced rectal cancer patients treated with neoadjuvant radiochemotherapy was analyzed.

GSTP1 is a polymorphic gene that encodes for a phase

II enzyme involved in detoxification of carcinogenic agents [18]. The over-expression of *GSTP1* protein has been reported in many solid and haematological human cancers such as breast cancer [19,20], multiple myeloma and Monoclonal gammopathy of undetermined significance [21]. The polymorphism A313G leads to a substitution of an isoleucine with a valine in the codon 105 (Ile105Val). This amino acid is located near the hydrophobic binding site for the electrophile substrate; thus, the protein has an altered affinity with the electrophile substrate and therefore a reduced catalytic activity [22], while the allele G confers a higher catalytic efficiency for polycyclic aromatic hydrocarbon diol epoxides [23]. The demonstration of differential effects of the two alleles is therefore translated in a differential outcome for heterozygous gene (GA), as compared to the homozygous. The correlation between *GSTP1* (A313G) and a higher predisposition to develop rectal cancer has been reported [24,25], as well as a correlation with an intensified radiochemotherapy and toxicity [13,26]. The results regarding a possible correlation between *GSTP1* (A313G) and the outcome after radiotherapy or radiochemotherapy in many tumours are controversial [27–30]. Lastly polymorphisms of *GSTP* gene have already been studied in rectal cancer with promising but still conflicting results.

In an extensive pharmacogenomic analysis on a subgroup of patients of the INT-0144 trial, Bohanes et al. [15] analyzed 18 polymorphisms in 16 gene finding a significant association between *GSTP1* (A313G) and overall survival and disease-free survival. Patients with the genotypes AG and AA had higher 3- and 5-year disease-free survivals and overall survivals than the low activity genotype GG in a range of 5%–12% and 4%–13% for the disease-free survival, 1%–5% and 8%–14% for the overall survival, respectively.

In the present study, a significantly higher rate of pathological complete response was shown for AA genotype compared to other genotypes (AG and GG). Lower survival of GG haplotype patients was found when compared to the

AA+AG patients. After 83 months of median follow-up, 5- and 8-year cancer-specific survivals were significantly better for AA+AG patients as compared to GG patients; while overall survivals had only a trend towards significance.

The results of the two studies, highlights the importance of rate of response to treatment, according to genetic parameters as a key step in the way of the personalization of treatment, due to the reported correlation between pathological complete response and longer survival [8].

Cecchin et al. [31] analyzed 25 polymorphisms in 16 genes in a population of rectal cancer patients treated with neoadjuvant radiochemotherapy, fluoropyrimidine alone or in combination and reported no association between *GSTP1* (A313G) and the TRG. Different treatment regimens, histopathological evaluation (Dworak scale vs. Mandard criteria) and outcome measurements may have contributed to these inconsistencies with our results.

XRCC1 is one of the most important genes of the base excision repair (BER) pathway. The encoded protein interacts with the DNA polymerase and the DNA ligase III in response to the damage of single DNA strand caused by the interaction with ionizing radiations, reactive oxygen intermediate and methylating agents. The substitution of a guanine with an adenine in position 28152 (G28152A) in exon 10 of the gene encoding for *XRCC1* leads to a substitution of an arginine with a glutamine in the codon 399 (Arg399Gln) and it is associated with a lower DNA repair ability [32,33].

In our series, no correlation between *XRCC1* (G28152A) and the rate of pathological complete response and survival has been found. Our results are in accordance to the findings by Balboa et al. [34], where the mutation was not able to predict the response after neoadjuvant radiochemotherapy with fluoropyrimidine-based chemotherapy in locally advanced rectal cancer patients. In contrast, Paez et al. [16] analyzed 9 SNPs in 128 rectal cancer patients, showing that *XRCC1* (G28152A) was predictive for a longer progression-free survival. Interestingly, the other analyzed SNPs including *GSTP1* (A313G) were not correlated to pathological stage and survival. These results could reflect the complexity of genes interaction.

Our study present some limits such as the retrospective nature and the small study population. On the other hand, our series underwent homogeneous treatment and the results were analyzed after long-term follow-up. Furthermore, our patients were not treated with oxaliplatin, used in some trials in combination with fluoropyrimidine, a possible confounding factor due to the known metabolic correlation with *GST*-genes

family [26,35].

In conclusion, more attention should be paid to this new approach of personalized radiotherapy in the future. Further trials are needed to evaluate new combinations of treatment, or the effect of a different total dose or altered fractionation on the basis of the genetic mutations.

The long-term results of our study suggest the possibility to use *GSTP1* (A313G) SNP to identify patients with different sensitivity to neoadjuvant radiochemotherapy. Mutated gene predicted for low rate of pathological complete response and poor survival. Future studies should confirm these results in a larger cohort of patients and evaluate the effects of an intensification of treatment in patients more sensitive to radiations. The upcoming evidence supporting the genetic basis of the radiosensitivity should arise the interest in deepen the study of these mechanisms with the aim of a personalized radiotherapy.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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