Original paper

Single nucleotide polymorphisms of *XRCC3* gene in hepatocellular carcinoma – relationship with clinicopathological features

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Recent studies support the involvement of XRCC3 gene polymorphisms in carcinogenesis. Our study focuses on the identification of polymorphic variants of XRCC3 in hepatocellular carcinoma (HCC) and an analysis of the relationship between these polymorphic variants and clinicopathological (including the genotype specific risk) and survival characteristics. Fifty cases of HCC were genotyped using molecular biology techniques for Thr241Met, rs861539 (c.722C>T) and 5'-UTR, rs1799796 (c.562-14A>G) polymorphisms. Statistical analysis was based on $\chi 2$, Fisher's, logistic regression (odd ratio – OR), and log-rank tests. Statistically significant differences were shown only for rs1799796 A>G and tumour grade, between wild type (AA) and heterozygote (AG) genotypes, and wild type (AA) and heterozygote & homozygote (AG & GG) genotypes. The logistic regression analysis found an OR of rs1799796 polymorphism occurrence in HCC related to tumour grade. The statistical analysis revealed, for the rs861539 C>T polymorphism, a better survival only for the homozygote genotype (TT) compared to the heterozygote (CT), and for rs1799796 A>G polymorphism, a longer survival for the wild type (AA) compared to heterozygote (AG) and to heterozygote & homozygote (AG & GG) genotypes, respectively. Our results suggest that XRCC3 gene SNPs could influence the tumour aggressiveness expressed by tumour grade.

Key words: XRCC3 gene, single nucleotide polymorphism, hepatocellular carcinoma, tumour grade, survival.

Introduction

Hepatocellular carcinoma (HCC) is the most frequent primary liver tumour, while it is the fifth most common cancer in men (7.5%) and the ninth in women (3.4%) [1]. Over 80% of cases are recorded in men, the sex ratio usually averaging between 2:1 and 4:1 [1, 2]. Large variations in incidence and mortality suggest a role of genetic and environmental factors in the pathogenesis of this type of cancer [2, 3].

Solid scientific evidence shows the effect of chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) in liver carcinogenesis [4, 5]; other risk factors are alcohol, liver cirrhosis, hemochromatosis, toxic exposure (e.g. aflatoxin), and non-alcoholic steatohepatitis [6, 7].

Recent studies in molecular epidemiology support the involvement of genetic factors in the carcinogenetic process of HCC [8], with DNA repair abnormalities playing a major role. Nucleotide excision repair is the most commonly used mechanism and represents a major defence pathway against damage caused by ionising radiation, highly chelating agents, and endogenous metabolic factors [9].

XRCC3 (*X-Ray Cross Complementing Group 3*) is a gene involved in DNA nucleotide excision repair.

Mutations and polymorphisms of this gene have an influence on the structural and functional particularities of nucleic acids, while incapacitation of *XRCC3* may increase the risk of developing various malignant neoplasms and congenital defects, and may determine the reduction in lifespan of the entire organism [10].

XRCC3 is located on chromosome 14q32.3, spans 17 kb, and includes 7 exons coding a 37 kDa protein, which consists of 346 amino acids [11, 12]. The most commonly investigated single nucleotide polymorphism (SNP) of *XRCC3* at the level of nitrogen bases is the replacement of cytosine by thymine in codon 241 of exon 7 and consequently the replacement of threonine by methionine in the encoded protein [13]. The SNP changes the function of the protein [14]. Three genotypes of this SNP are identified, referred to as wild type (CC), heterozygote (CT), and homozygote (TT) [3].

In the DNA double-strand break repair, XRCC3 protein intervenes both in cross-link and homologous recombination repair mechanisms, representing a key component of the pathway, because it is a homolog of the RAD51 protein [15] with which it interacts [16]. The association between XRCC3 and RAD51 proteins facilitates the synthesis of a nucleoprotein filament, which represents the primordial vector both for homologous and heterologous recombination [15, 17, 18].

Another *XRCC3* polymorphism occurs in area 5´UTR and is structurally expressed exclusively at gene level. It consists of the substitution of adenine with guanine, and it is located in an uncoding segment [19]. For this SNP there are three genotype variants: wild type (AA), heterozygote (AG), and homozygote (GG) [20].

Research on alterations in DNA repair genes is motivated by evidence showing that deficiencies in their function cause genomic instability and promote tumour development [21, 22, 23, 24].

Our investigation was focused on the identification of polymorphic variants of *XRCC3* expressed by HCC. We also analysed the relationship between the detected polymorphic variants and clinicopathological characteristics (including the genotype specific risk) and survival, with the aim to associate the SNP expression with tumour behaviour.

Materials and methods

Patients

The study group included 50 consecutive cases diagnosed with HCC between 1 January 2009 and 31 December 2011 at the Department of Pathology of the County Clinical Emergency Hospital "Sf. Spiridon", Iaşi. Thirteen patients were female and 37 were male, with a mean age ±SD of 64.62 ±8.21 years.

Among all patients, 24 had been treated with surgical resection (segmentectomy or lobectomy), whereas 26 had received local ablative therapy (radiofrequency ablation [RFA] – 24 cases, percutaneous ethanol injection [PEI] – 2 cases).

The examined material consisted of surgical specimens routinely processed for pathological examination.

All cases were histologically reassessed including HCC differentiation and stage. According to the TNM system, the cases were classified as follows: nine cases as T1, 19 cases as T2, 21 cases as T3, and one case as T4. Regarding tumour grade, 23 cases were well differentiated, 18 cases moderately differentiated, and nine poorly differentiated. The cirrhotic background was present in 22 cases (seven with HBV infection, 10 with HCV infection, and five of alcoholic aetiology). Cirrhosis was absent in 28 cases, among which six had chronic HBV and nine chronic HCV infection, five showed steatosis, and eight had non-alcoholic steatohepatitis.

Follow-up was performed every three months during the first two years and every six months later on [25]. According to follow-up data, patients' survival ranged from 0 months (one patient died during the first month after surgery) to four years, with an average of 26.5 months.

The study was approved by the Ethics Committee of the "Grigore T. Popa" University of Medicine and Pharmacy, based on the patients' informed written consent for the use of the biological material for research.

Genotyping

The molecular biology techniques (DNA extraction, PCR amplification, and pyrosequencing) were performed in the Laboratory of Molecular Pathology, University Hospital "Santa Chiara", University of Pisa, Italy. For each case, we used for the extraction of genomic DNA two sections of formalin-fixed, paraffin-embedded liver tumour tissue (corresponding to surgical specimens), with a thickness of 10 μm; the technique was performed in absolutely sterile conditions. The sections were initially dewaxed, and the steps of genomic DNA extraction (Macherey-Nagel GmbH & Co. KG, July 2009/Rev. 10) from tissues were followed. The primer pairs used for amplification of the XRCC3 Thr241Met (rs861539) locus were as follows: sense primer: 5'-GGCCAGGCATCTGCAGTC-3', antisense primer: 5'-CAGCACAGGGCTCTG-GAA-3'; and for amplification of the XRCC3 5UTR (rs1799794) locus were as follows: sense primer: 5'-GCCTGTTAAACCAAGTTCTCAGC-3', antisense primer: 5'-GGAAGCAGAGTGTCCACTGAC -3' (PrimerQuest® program, IDT, Coralville, USA). Re-

al-Time Polymerase Chain Reaction (RT-PCR) amplifications were carried out in a total volume of 50 μl containing 27.3 μl water, 10 μl 5X PCR buffer (containing MgCl2) (Takara, Shiga, Japan), 1.5 μl (mM) of each dNTP (Takara, Shiga, Japan), 2.5 μl Eva Green, 2 μl of each primer (10 μM/μl), 3 μl genomic DNA as a template, and 0.3 μl (5U/μl) Taq polymerase (Takara, Shiga, Japan). We used the RT-PCR Machine Rotor Gene 6000 Corbett Research System (Qiagen, UK). The RT-PCR conditions were as follows: predenaturation at 94°C for 3 min; 40 cycles of denaturation at 94°C for 20 s, annealing at 63°C for 30 s, and extension at 72°C for 30 s; final extension was performed at 60°C for 5 min. The PCR products were directly sequenced using the PyroMarkQ96 System (Qiagen, UK). Each step was performed with internal control, while a 5% randomly selected fraction of the specimens was reanalysed for verification of the method. The results were identical in all cases.

Reference sequences used for *XRCC3* gene were NC_000014.9 for genomic, NM_001100118 for cDNA, and NP_001093588.1 for protein coordinates. HGVS (Human Genomic Variation Society) nomenclature was used.

Statistical analysis

The MedCalc software package (MedCalc Software, Ostend, Belgium) was used for statistical analysis. The relationship between various genotypes or alleles and the clinicopathological features was analysed using the χ^2 test and Fisher's exact test.

The risk of SNP expression in HCC associated with different clinicopathological features (HCV infection, HBV infection, cirrhosis, HCV/HBV infections associated with cirrhosis, tumour stage, tumour grade) was assessed using logistic regression, as the odds ratio (OR). This risk was defined as SNP risk in relation to different HCC-associated parameters.

For survival analysis we performed the log-rank test; statistical significance was accepted when $p < 0.05$. The survival curves were plotted using the Kaplan-Meier model.

Results

The sequencing of *XRCC3* gene in the 50 HCC detected Thr241Met, rs861539 (c.722C>T), and 5'-UTR, rs1799796 (c.562-14A>G) polymorphisms. The position of the SNPs and their correlation with the exon-intron parameters of *XRCC3* are shown in Table I. The genotype and allele frequency of *XRCC3* polymorphisms are presented in Table II. The relationship between the genotypes of the *XRCC3* gene SNPs and the clinicopathological features are summarised in Table III.

The statistical analysis indicated no association between all three rs861539 C>T genotypes and the clinicopathological characteristics (Table III). However, significant differences were shown for rs1799796 A>G and tumour grade (well differentiated versus moderately and poorly differentiated), between wild type (AA) and heterozygote (AG) genotypes ($p_{\chi^2} = 0.02$, $p_{Fisher2\times2} = 0.01$), and wild type (AA) and heterozygote & homozygote (AG&GG) genotypes ($p_{\chi^2} = 0.03$, $p_{Fisher2\times2} = 0.02$) (Table III).

The logistic regression analysis performed to assess the SNPs risk in relation to HCC-associated parameters revealed no risk related to the presence or absence of HCV infection, HBV infection, cirrhosis, HCV/HBV infections associated with cirrhosis, and tumour stage. Only for rs1799796 A>G and tumour grade (well differentiated versus moderately and poorly differentiated) the OR was confirmed (AA versus AG: OR [95% CI] = 0.16 [0.03 – 0.71], $p_{OR} = 0.0162$; AA versus AG&GG: OR [95% CI] = 0.22 [0.06 – 0.77], $p_{OR} = 0.0182$).

The survival analysis showed significant differences between the cases treated by surgery and those treated by local ablative therapy ($p = 0.0059$) (Fig. 1). For the rs861539 C>T polymorphism, the Kaplan-Meier curves revealed no differences between wild type (CC) and heterozygote (TC) genotypes ($p = 0.18$) (Fig. 2A), or between wild type (CC) and heterozygote & homozygote (TC&TT) genotypes ($p = 0.47$) (Fig. 2B); a better survival was noted only for the homozygote genotype (TT) compared to the heterozygote genotype (TC) ($p = 0.04$) (Fig. 2C). On the other hand, the survival analysis for the rs1799796 A>G polymorphism indicated a longer survival for the wild type (AA) compared to heterozygote (AG) and to heterozygote & homozygote (AG&GG) genotypes, respectively ($p = 0.0009$, $p = 0.0025$) (Fig. 2D, 2E); no differences were found between homozygote (GG) and heterozygote (AG) genotypes ($p = 0.32$) (Fig. 2F).

Discussion

It is unanimously accepted that liver carcinogenesis is closely related to the inflammatory background, which progressively leads to the cirrhotic state charac-

Table I. Position of SNPs and their correlation to exon-intron parameters of *XRCC3* gene

Gene	rsNo.	Genomic Position	HGVS NAME	Protein effect	Location
XRCC3	861539	g.103699416	c.722C>T	p.Thr241Met	EXON
	1799796	g.103699590	c.562-14A>G	n/a	INTRON

Table III. Clinicopathological features in relation to genotypes of the *XRCC3* gene SNPs in HCC patients

Gene		*XRCC3* rs861539 C>T			*XRCC3* rs1799796 A>G		
Genotypes		CC vs. TC	CC vs. TT	CC vs. TC&TT	AA vs. AG	AA vs. GG	AA vs. AG&GG
Variables				number of cases			
Age group							
< 65	28	10/14	10/4	10/18	16/10	16/2	16/12
> 65	22	11/9	11/2	11/11	14/5	14/3	14/8
pχ^2		0.56	0.71	0.46	0.59	0.94	0.23
Gender							
Female	13	5/6	5/2	5/8	8/2	8/3	8/5
Male	37	16/17	16/4	16/21	22/13	22/2	22/15
pχ^2		0.86	0.95	0.97	0.52	0.33	0.84
HBV infection							
HBV (+)	13	6/5	6/2	6/7	7/4	7/2	7/6
HBV (–)	37	15/18	15/4	15/22	23/11	23/3	23/14
pχ^2		0.86	0.77	0.97	0.90	0.81	0.84
HCV infection							
HCV (+)	19	11/8	11/0	11/8	11/6	11/2	11/8
HCV (–)	31	10/15	10/6	10/21	19/9	19/3	19/12
pχ^2		0.38	0.06	0.13	0.91	0.72	0.95
Cirrhosis							
Cirrhosis (+)	22	10/9	10/3	10/12	14/6	14/2	14/8
Cirrhosis (–)	28	11/14	11/3	11/17	16/9	16/3	16/12
pχ^2		0.79	0.71	0.88	0.91	0.83	0.86
HBV/HCV-infected cirrhotic cases versus non-cirrhotic, non-infected cases							
HVB/HCV (+) cirrhosis (+)	17	9/6	9/2	9/8	11/5	11/1	11/6
HVB/HCV (–) cirrhosis (–)	13	4/6	4/3	4/9	9/3	9/1	9/4
pχ^2		0.56	0.54	0.39	0.95	0.54	0.89
Tumour stage							
T1+T2	28	12/12	12/4	12/16	19/6	19/3	19/9
T3+T4	22	9/11	9/2	9/13	11/9	11/2	11/11
pχ^2		0.97	0.95	0.88	0.24	0.72	0.32
Tumour grade							
WD	23	9/10	9/4	9/14	18/3	18/2	18/5
MD+PD	27	12/13	12/2	12/15	12/12	12/3	12/15
pχ^2		0.79	0.57	0.92	0.02	0.72	0.03

*χ^2 – Chi-square test (*Fisher's exact test provides similar data), WD – well differentiated, MD – moderately differentiated, PD – poorly differentiated; (+) – present, (–) – absent*

terised by persistence of inflammation and stimulation of hepatocellular proliferation [26, 27]. In the context of cirrhosis, DNA suffers oxidative damage followed by activation of repair mechanisms, often accompanied by genetic alterations translated into mutations. Moreover, the rate of constant activation of cell division increases, and the odds for occurrence of DNA replicative errors favour the appearance of mutations involved in initiation and development of liver cancer.

Mainstream publications include numerous studies proving that rs861539 polymorphism of the *XRCC3* gene influences susceptibility of developing several types of cancer, namely: oesophageal [28, 29], gastric [30, 31], breast [32, 33, 34, 35], colorectal [36, 37, 38], urinary bladder [39], ovarian [40], thyroid [41], prostate [42], liver [3, 43, 44, 45, 46, 47, 48, 49, 50], and glioma [20, 51]. On the other hand, there is little information about the functional consequences of the presence of polymorphism rs1799796 because it is scarcely investigated. Published data are contradictory; certain studies support the involvement of this polymorphism in the evolution of ovarian cancer in patients in China [40] and initiation of the carcinogenetic process in the skin [52], while others reject it as regards breast [35, 53, 54] and thyroid cancer [41]. To the best of our knowledge, there is no study focused on liver tumours besides our preliminary results communicated in 2015 at the European Congress of Pathology [24].

The importance of the epidemiological data about *XRCC3* polymorphism and its association with cancer risk is sustained by a number of case-control designed studies [55] that offer important information regarding the frequency of *XRCC3* polymorphism in different malignancies and in a control population. Unlike these studies, our research did not focus on *XRCC3* polymorphism epidemiology, so this work does not refer to a control group. Starting from the assumption that some variations in *XRCC3* may contribute to HCC susceptibility, our scientific interest was strictly oriented towards the relationship between the *XRCC3* gene SNPs and clinicopathological features of HCC, aiming to investigate whether SNPs influence tumour behaviour.

A particular aspect of our study is the presence of HCC in a relatively high number of non-cirrhotic livers, at odds with current large-scale epidemiological data [2]. There are two possible explanations for this discrepancy. First, selection of patients who have undergone curative resection or ablation for HCC in our study may have generated a bias towards a particular population of non-cirrhotic HCC patients. Second, regional genotype characteristics for both HBV and HCV infections associated with a high risk of development of HCC [56, 57] in the context of historical [56, 58] and current [59, 60] unusually high incidence of HBV and HCV in Romania may explain the increased incidence of HCC in non-cirrhotic patients. While the specific mechanisms involved in this discrepancy are beyond the scope of our present study, further investigation is warranted.

The analysis of rs861539 polymorphism yielded completely different results from those obtained by other researchers. Our study indicates a lack of correlation between the expression of the examined SNP and patients' age and sex, presence of HBV or HCV infection, cirrhotic background of primary liver tumour, tumour stage, tumour grade, and therapeutic management. Moreover, the absence of a relationship of the polymorphic variants with HBV/HCV-infected cirrhotic cases compared to non-cirrhotic, non-infected ones does not sustain a possible involvement of SNPs in HCC pathogenesis. Furthermore, we did not find a significant association between rs861539 polymorphism and HCC risk.

Table II. Genotype and allele frequency of rs861539 C>T and rs1799796 A>G polymorphisms in HCC patients

XRCC3 SNPs	HCC PATIENTS	
rs861539 C>T	#	%
CC	21	42
TC	23	46
TT	6	12
TC&TT	29	78
C	65	65
T	35	35
rs1799796 A>G	#	%
AA	30	60
AG	15	30
GG	5	10
AG&GG	20	40
A	75	75
G	25	25

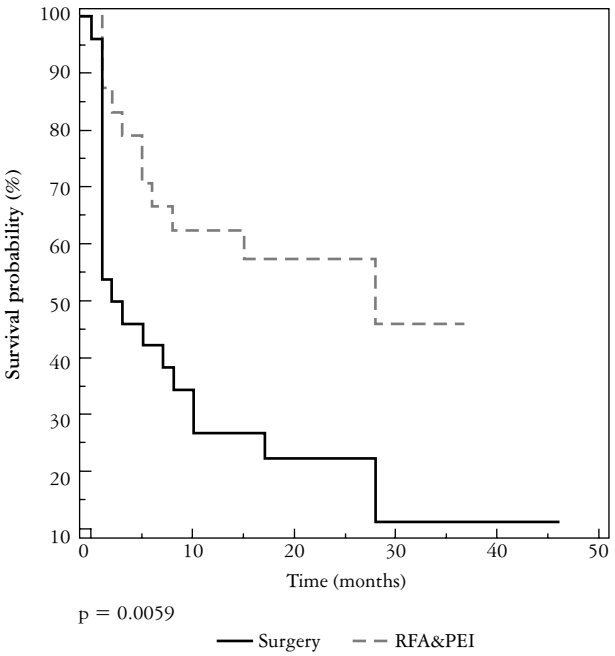

Fig. 1. Kaplan-Meier curve: association between therapeutic approaches in HCC patients and overall survival; the p values are for the log-rank test

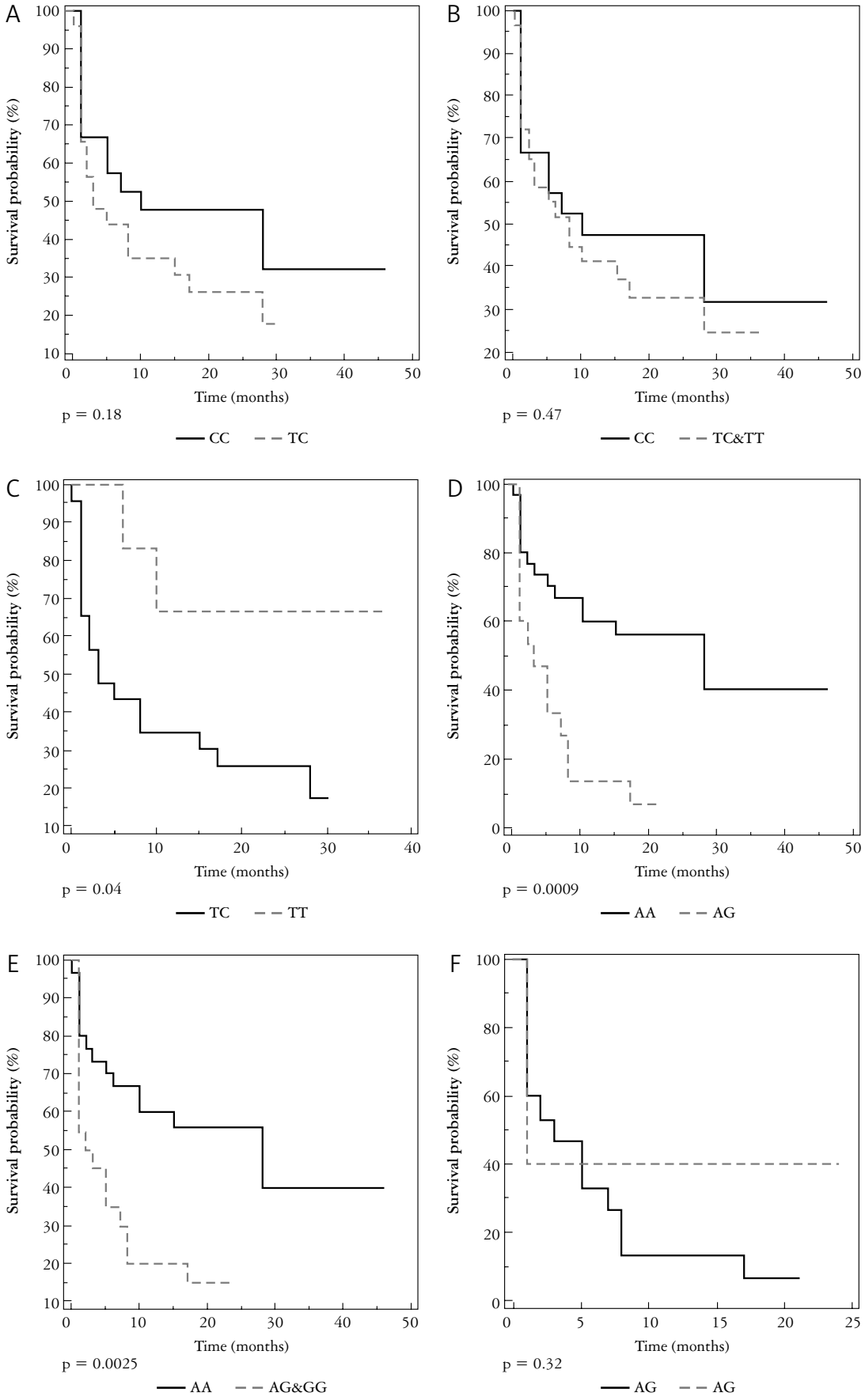

Fig. 2. Kaplan-Meier curves: association between rs861539 genotype variants and overall survival (A, B, C); association between rs1799796 genotype variants and overall survival (D, E, F); the p values are for the log-rank test

The presence of rs861539 polymorphism seems to influence survival favourably only for patients who display a mutant genotype, homozygote versus heterozygote. This finding opens generous perspectives for interpretation, because until now it was believed that patients with wild-type genotype of rs861539 polymorphism have a better prognosis than those with mutant genotype [47, 48, 49]. Therefore, we consider that rs861539 polymorphism may not represent a critical element in the progression of liver carcinogenesis lacking a decisive interference with clinical, histological, and survival parameters.

The broad view on the involvement of gene polymorphisms in the pathogenic mechanisms of HCC is based on the results obtained for rs1799796 polymorphism. It is worth re-emphasising that this polymorphism is sparsely investigated in tumour pathology. No correlations were found between rs1799796 polymorphism and the following clinicopathological characteristics: age, sex, viral infection, cirrhotic background – independent or connected to the presence or absence of HBV/HCV infection, tumour stage, and therapeutic management. However, statistical analysis showed an association of SNP expression with histological grade of the tumour. This finding indicates that there are differences between the behaviour of wild type and mutant genotypes because the latter is associated with higher aggressiveness, translated into moderate or poor differentiation grade.

In addition, we demonstrated an OR of rs1799796 polymorphism occurrence in HCC related to tumour grade, where the heterozygote (AG) and heterozygote & homozygote (AG&GG) genotypes were associated with the development of moderately and poorly differentiated histological variants.

Moreover, the survival analysis showed a better survival rate of wild type patients as opposed to mutant patients. Thus, rs1799796 polymorphism could be a risk factor for the development of more aggressive HCC types and may also harbour potential prognostic value in monitoring patients' evolution.

Unfortunately, the small number of cases represents a definitive limitation, which renders the formulation of broadly covering conclusions difficult. However, we believe that the novelty of our results regarding supports the understanding of SNP involvement in tumour development and behaviour.

In conclusion, our findings suggest that *XRCC3* gene SNPs may influence tumour aggressiveness expressed by tumour grade and survival. We consider our work as a preliminary study, which allowed the collection of supplementary information on the polymorphic variants of *XRCC3* and the identification of key issues regarding their association with the clinicopathological characteristics of HCC.

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