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The additive effect of *p53* Arg72Pro and *RNASEL* Arg462Gln genotypes on age of disease onset in Lynch syndrome patients with pathogenic germline mutations in *MSH2* or *MLH1*

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Abstract

p53 and the prostate-cancer-susceptibility gene *RNASEL* are tumour suppressor genes involved in apoptosis. We have previously reported that the common, functionally different variants Arg72Pro in *p53* and Arg462Gln in *RNASEL* are associated with the age of disease onset of colorectal cancer in Lynch syndrome patients. To assess the combined effect of both variants, we screened 246 unrelated Lynch syndrome patients with a pathogenic germline mutation either in *MSH2* ($n = 138$) or in *MLH1* ($n = 108$) and colorectal cancer as first tumour, and 245 healthy controls. The global log rank test revealed significant differences in the age of disease onset for the genotypes of each variant ($p = 0.0176$ for *p53* and $p = 0.0358$ for *RNASEL*) and for the combined genotypes of both variants ($p = 0.0174$). The highest difference in median age of disease onset was seen between homozygotes for the wild-types in both genes (42 years [range 22–75]) and homozygotes for the variant alleles in both genes (30 years [range 26–47]). A multivariate Cox regression model indicated that only the *p53* and *RNASEL* genotypes had a significant influence on age of disease onset ($p = 0.016$ for *p53* and

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$p = 0.014$ for *RNASEL*) in an additive mode of inheritance, and that the effects of both variants are purely additive, which supports the notion that the *p53* and *RNaseL* pathways do not interact. These findings may be relevant for preventive strategies in Lynch syndrome.

Keywords: *p53* Arg72Pro; *RNASEL* Arg462Gln; Hereditary nonpolyposis colorectal cancer (HNPCC); Mismatch repair (MMR) system; Age of disease onset (AO)

1. Introduction

Lynch syndrome, also named *hereditary nonpolyposis colorectal cancer* (HNPCC), is one of the most common cancer susceptibility syndromes, with an autosomal dominant mode of inheritance. HNPCC is mostly caused by germline mutations in the DNA mismatch repair (MMR) genes *MSH2* and *MLH1* [1], and is characterised by highly microsatellite-unstable (MSI-H) tumours [2]. Mutation carriers are at high risk for developing colorectal carcinomas (CRC), as well as extracolonic neoplasias [3,4]. Therefore, a specific surveillance program is recommended. In addition to an incomplete penetrance of about 80% for CRC and to the broad tumour spectrum, a wide variety of age of disease onset (AO, ranging from 16 to 90 years) has been described [5], suggesting the existence of modifying genetic or environmental factors.

Several studies have assessed whether or not the common *p53* sequence variant Arg72Pro is involved in the development of cancer. Since both alleles differ in their susceptibility to degradation by human papilloma virus (HPV) E6 protein [6], the association between these alleles and cancer risk has been studied in cervical cancer and other tumours, with controversial results [6–10]. Recently, it has been shown that the variants at codon 72 of *p53* are functionally distinct *in vitro*, whereby the Arg72 variant induces apoptosis at least five times more efficiently than the Pro72 variant [11]. Studies in the *MLH1*-deficient colorectal cancer cell line HCT-116 and in knock-out mice provided evidence for a cooperation between the MMR system and p53 in tumourigenesis [12–15]. We have shown that the *p53* Arg72Pro sequence variant was significantly associated with AO in 167 unrelated HNPCC patients with pathogenic MMR germline mutations and CRC as first tumour manifestation. In contrast, no association between *p53* genotype and AO was found in 126 patients with sporadic microsatellite-stable CRC, supporting the notion of cooperation between the MMR system and p53 in tumourigenesis in humans [16].

RNASEL encodes an ubiquitously expressed endoribonuclease acting in the interferon regulated 2′-5′-linked oligoadenylates (2-5A) system. The 2-5A/RNase L-pathway is implicated in mediating apoptosis in response to either viral infections or several other external stimuli [17–20], and has been suggested as a tumour suppressor gene [17–19,21]. Recently, two different germline mutations in *RNASEL* (E265X and M1I) have been shown to cosegregate in two hereditary prostate cancer families. Tumour tissue from carriers of the E265X mutation showed loss of the wild-type *RNASEL* allele [21]. A third inactivating *RNASEL* germline mutation (c.471_474delAAAG) was found in Ashkenazi with prostate cancer [22]. Another variant, Arg462Gln, was found to be associated with both hereditary [23] and sporadic prostate cancer. Heterozygotes had about 1.5-fold increased risk, and glutamine homozygotes had more than double the risk of developing prostate cancer [24]. Furthermore, the variants at codon 462 were shown to be functionally distinct *in vitro*, whereby the glutamine variant had a 3-fold decrease in enzyme activity compared to the arginine variant [20,24], and was deficient in causing apoptosis in response to 2-5A, suggesting that this variant allows tumour cells to escape a potent apoptotic pathway [20]. A possible role for this sequence variant in any of the other common neoplasias was inferred [19]. Notably, other studies did not find any association between *RNASEL* Arg462Gln and prostate cancer risk [25–27]. Recently, we have shown a significant association between the *RNASEL* Arg462Gln sequence variant and AO in 251 unrelated HNPCC patients with pathogenic germline mutations in *MSH2* or *MLH1* and CRC as the first tumour manifestation [28].

To assess the effect of the combination of both sequence variants (i.e., Arg72Pro of *p53* and Arg462Gln in *RNASEL*) on the phenotype of HNPCC, we analysed 246 unrelated HNPCC patients with pathogenic germline mutations in *MSH2* or *MLH1* and CRC as the first tumour manifestation and 245 healthy controls.

2. Patients and methods

2.1. Patients

We have studied 246 unrelated HNPCC patients (151 male and 95 female) consecutively registered and analysed at the clinical centres of the German HNPCC Consortium in Bonn, Dresden, Munich–Regensburg, Heidelberg, Duesseldorf, and Bochum. Each centre gathered patients both locally and in the extended surroundings. Two hundred and forty patients met Bethesda criteria [29], while five of these met the revised Bethesda criteria [30]. One patient did not meet any of the revised Bethesda criteria. He suffered from colon cancer at age 62 and his brother was affected with colon cancer at age 53. To avoid possible familial or genetic factors inducing a correlation in family members (besides the *p53* and *RNASEL* genotype), we have included only one member per family. All 246 individuals studied were the nominal probands in their respective family. Microsatellite analyses and immunohistochemistry (IHC) analyses of mismatch repair protein expression of MSH2 and MLH1 were performed as screening method if tumour material was available. Aberrant findings such as MSI-H or lost or reduced expression of at least one MMR protein in IHC led to mutation screening. In patients who fulfilled Amsterdam I/II criteria [31] but tumour material was not available, mutation screening in *MSH2* and *MLH1* was performed. Inclusion criteria, clinical and molecular data of our registry, microsatellite analysis, IHC, and mutation screening are described in detail elsewhere [32–34]. All patients described here were carriers of germline mutations in either *MSH2* (138 patients) or *MLH1* (108 patients), predicted to be pathogenic due to their nature as protein truncating small insertions/deletions, large genomic rearrangements, nonsense or splice site mutations. To control for the false inclusion of non-pathogenic missense mutations in *MSH2* and *MLH1*, we completely excluded carriers of missense mutations. The first tumour manifestation in all 246 patients was a colorectal carcinoma revealed by medical history and histopathological examination. AO was defined as the time of histological tumour diagnosis. All patients gave written informed consent for study participation. 245 anonymous healthy blood donors from the Dresden Regional Blood Centre served as controls. The compiled data analysis of the patients and the controls is summarised in Table 1.

Tumours of 208 patients were MSI-H and two were microsatellite-stable (MSS), while in 36 cases tumour material was unavailable or could not be assessed. IHC revealed loss of MLH1 protein expression alone in the tumour cells in 73 patients, loss of MSH2 expression alone in 99 cases, and loss of both MMR proteins in one patient. In tumours of 19 patients no loss of MMR protein expression could be detected, whilst in 54 patients tumour material was unavailable or could not be assessed (Table 2).

Table 1
Compiled data analysis of the patients and controls

	MMR mutation carrier	Healthy controls
All	246	245
Gender		
Male	151	113
Female	95	132
MMR gene mutated		
MSH2	138	
MLH1	108	
Median age [years] of onset of CRC (range)	38 (13–75)	36 (18–65)[a]
Criterion		
Amsterdam I/II[27]	139	
Bethesda[25] 2	6	
Bethesda 2 and 4	11	
Bethesda 2, 3 and 4	37	
Bethesda 2, 3, 4 and 7	2	
Bethesda 2, 4 and 7	3	
Bethesda 3	1	
Bethesda 3 and 4	18	
Bethesda 3, 4 and 7	2	
Bethesda 4	21	
None	6[b]	

[a] Age.
[b] Five of these met the revised Bethesda criteria [30].

Table 2
Results of microsatellite analyses (MSI) and immunohistochemistry of 246 HNPCC patients in relationship to the location of the MMR gene mutation

MSI	Immunohistochemistry		*n*	Mutation	
	MLH1	MSH2		*MLH1* (*n*)	*MSH2* (*n*)
MSI-H	Neg	Pos	68	68	
MSI-H	Neg	Neg	1		1
MSI-H	Neg	n.r.[a]	1	1	
MSI-H	Pos	Neg	95		95
MSI-H	Pos	Pos	14	10	4
MSI-H	Pos	n.r.	3		3
MSI-H	n.r.	Neg	1		1
MSI-H	n.r.	Pos	1	1	
MSI-H	n.r.	n.r.	24	11	13
MSS	Pos	Pos	1	1	
MSS	n.r.	n.r.	1	1	
n.r.	Neg	Pos	4	4	
n.r.	Pos	Neg	3		3
n.r.	n.r.	n.r.	29	11	18
All			246	108	138

[a] n.r., no results available because tumour material was unavailable (most cases) or investigations were without conclusive results.

The results concerning the individual role of each sequence variant of a part of the 246 patients described here were published previously [16,28]. This study was approved by the respective local ethics committee in each of the participating centres.

2.2. Genotyping of p53 p.Arg72Pro and RNASEL p.Arg462Gln sequence variations

Genotyping of sequence variation in both genes was performed on genomic DNA isolated from peripheral blood leukocytes as previously described [16,28].

Briefly, for genotyping of *p53* codon 72 PCR-amplification (*Taq*-polymerase, Perkin-Elmer Applied Biosystems, Weiterstadt, Germany) of exon 4 of *p53* using primers 5′-TGAGGACCTGGTCCTCTGAC-3′ and 5′-AGAGGAA TCCCAAAGTTCCA-3′ [35], was performed. We used single base sequencing of PCR-products using the Thermo Sequenase Fluorescent Cycle Sequencing kit and Automated Laser Fluorescence (A.L.F.express) sequencing devices (both Amersham Pharmacia Biotech, Freiburg, Germany) as described by Kristensen et al. [36] in 491 samples. Sequencing was performed with the Cy5-labeled antisense primer 5′-ATACGGCCAGGCATTGAAGT-3′ [35] using reagents including only cytosine, guanine or both as dideoxynucleotide from the Thermo Sequenase Fluorescent Cycle Sequencing kit. Results were confirmed in 390 samples by digestion of PCR-products with endonuclease *Bst*U1 (restriction site: 5′...CG▼CG...3′, 3′... CG▲CG...5′), which specifically cleaves the allele coding for Arg72 (CGC) but not the allele coding for Pro72 (CCC). Fragments were analysed on an agarose gel. Another 81 samples were confirmed by direct Sanger sequencing on an ABI 3730 DNA Analyser (Applied Biosystems). PCR was performed using the primers described above. Sequencing was performed with the reverse primer using ABI PRISM® BigDye® Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase v3.0 and v3.1 (Applied Biosystems). After an initial denaturation step at 95 °C for 1 min, amplification was performed using 30 cycles of denaturation (96 °C for 10 s), annealing (52 °C for 5 s), and extension (60 °C for 3 min). Sequences were analysed with the ABI PRISM® AB DNA Sequencing Analysis Software v 5.1.1 (Applied Biosystems).

Genotyping of *RNASEL* codon 462 of all 491 samples was performed by use of a LightCycler™ (Roche Diagnostics). Briefly, for PCR amplification we used 5′-TGTGTGTGTCACCCTC-3′ and 5′-TGGGGACTCAC CTATTAAG-3′ as forward and reverse primers. The sequence of the anchor probe was 5′-GGACAAGTG TAGTTCTTGAACAGCCTTAAAT X-3′, labeled with fluorescein, and the sequence of the sensor probe, labeled with LC-Red 640, was 5‘-LCRed 640-AGATGACAG GACATTTCGGGC P-3′. For PCR the LightCycler-Fast-Start DNA Master Hybridization Probes Kit (Roche Diagnostics) was used. After an initial denaturation step at 95 °C for 10 min, amplification was performed using 32 cycles of denaturation (94 °C for 5 s), annealing (58 °C for 20 s) and extension (72 °C for 15 s). After amplification was completed, a final melting curve was recorded. Initially, the probe was cooled down to 40 °C at 20 °C/s and then slowly heated (0.2 °C/s) until a temperature of 90 °C was reached. Fifty-three of all 491 genotyped probes were randomly chosen and LightCycler results were confirmed using direct Sanger sequencing as described by genotyping of *p53*. PCR was performed using the primers described above and sequencing was performed with the reverse primer.

2.3. Statistical methods

The age of onset of the first colorectal cancer was analysed by the Kaplan–Meier (product-limit) method. The log rank test was applied to compare the age of onset between genotype groups. A global comparison across all three groups in each single gene (i.e., *p53* and *RNASEL*) and across all nine possible combinations of genotypes from both genes was performed. Multivariate Cox regression analysis was used to assess the effect of several covariates (*MSH2* or *MLH1* germline mutation, gender, *p53* and *RNASEL* genotype) on age of onset. The hazard function in this model was defined as $h(t) = h_0(t)\exp(\beta_1 G + \beta_2 S + \beta_3 R_A + \beta_4 P_A + \beta_5 R_D + \beta_6 P_D + \beta_7 R_A P_A)$. "G" is an indicator variable for the location of the MMR gene defect (coded as 1 for *MLH1* and 2 for *MSH2*). "S" represents an indicator variable for gender (coded as 1 for male and 2 for female). "R_A" represents the genotypes at codon 462 of *RNASEL* (i.e., being 0 for the Arg/Arg, 1 for the Arg/Gln and 2 for the Gln/Gln genotype) which is a measure for the additivity of alleles. "P_A" represents the genotypes at codon 72 of *p53* (i.e., being 0 for the Arg/Arg, 1 for the Arg/Pro and 2 for the Pro/Pro genotype) which is a measure for the additivity of alleles. "R_D" and "P_D" measures departure from additivity (i.e., dominance) and is coded as 1 for the Arg/Gln genotype and as 0 for the Arg/Arg and Gln/Gln genotypes in *RNASEL*, and as 1 for the Arg/Pro genotype and as 0 for the Arg/Arg and Pro/Pro genotypes in *p53*, respectively. The product "$R_A P_A$" allows for a possible interaction between the two genes. The χ^2 test and Fisher's exact test were used to evaluate the homogeneity of genotype frequency distributions among patients and controls. Genotype frequencies in all groups were cross-checked with the Hardy–Weinberg expectations also by means of the χ^2 test. *p*-Values below 0.05 were considered significant. The statistical software package SPSS 10.0.7 was used for all statistical data analyses.

3. Results

We did not observe any significant differences in frequency of genotypes among patients and controls (Table 3), neither in the *p53* Arg72Pro variant ($p = 0.991$, χ^2 test)

Table 3
Distribution of *p53* Arg72Pro and *RNASEL* Arg462Gln sequence variants among cases and controls and median age of onset of the cases

Group	All *n*	*p53* codon 72				*RNASEL* codon 462			
		Arg/Arg *n* (%)	Arg/Pro *n* (%)	Pro/Pro *n* (%)	*P* value	Arg/Arg *n* (%)	Arg/Gln *n* (%)	Gln/Gln *n* (%)	*P* value
Controls	245	150 (61.2)	78 (31.8)	17 (7.0)		87 (35.5)	122 (49.8)	36 (14.7)	
Patients	246	152 (61.8)	77 (31.3)	17 (6.9)	0.991[a]	90 (36.6)	123 (50.0)	33 (13.4)	0.912[a]
AO[b]	38	39	35	33		40	37	34	
Range[c]		13–75	17–67	26–48		17–75	13–69	20–49	
95% CI[d]		37–41	32–38	29–37	0.0176[e]	37–43	34–40	32–36	0.0358[e]

[a] χ^2 test of genotype distribution in mutation carriers vs. controls.
[b] AO, median age of onset of CRC in patients in years.
[c] Years.
[d] 95% Confidence interval in years.
[e] Global log rank test (Kaplan–Meier analysis).

nor in the *RNASEL* Arg462Gln variant ($p = 0.912$, χ^2 test). Genotype frequencies in both genes in patients and controls were in accordance with those previously reported in Caucasians [9,10,23,24]. No significant deviations from the Hardy–Weinberg equilibrium were noted. Table 4 shows the distribution on the nine possible combinations between the two variants analysed in patients and controls, which was in accordance with those previously expected from the frequencies of both single genotypes in patients ($p = 0.574$, Fisher's exact test) as well as in controls ($p = 0.157$, Fisher's exact test). No significant differences in the combinations of both genotypes among patients and controls were observed ($p = 0.649$, Fisher's exact test).

In all 246 patients the median age of onset was 38 years (range 13–75). The median AO was 39 years (range 13–75, 95% confidence interval 37–41) for *p53* Arg/Arg, 35 years (range 17–67, 95% confidence interval 32–38) for *p53* Arg/Pro, and 33 years (range 26–48, 95% confidence interval 29–37) for *p53* Pro/Pro individuals. For the *RNASEL* Arg462Gln variation the median age of onset was 40 years (range 17–75, 95% confidence interval 37–43) for patients with Arg/Arg genotype, 37 years (range 13–69, 95% confidence interval 34–40) for Arg/Gln, and 34 years (range 20–49, 95% confidence interval 32–36) for those with Gln/Gln genotype (Table 3). In a global comparison, the age of onset was significantly different between the three p53-genotypes ($p = 0.0176$, log rank) as well as between the three RNASEL-genotypes ($p = 0.0358$, log rank; see Fig. 1 above and middle: Kaplan–Meier analysis).

The median AO with range and 95% confidence interval for the nine possible combinations between the Arg72Pro variant in *p53* and the Arg462Gln variant in *RNASEL* in patients and controls is also shown in Table 4. The difference in median AO among homozygous carriers of the wild-types (42 years) and homozygous carriers of the variant alleles (30 years) in both genes was 12 years. In the global comparison, the age of onset was significantly associated with the combination of genotypes of both genes ($p = 0.0174$, log rank; see Fig. 1 bottom: Kaplan–Meier analysis).

Multivariate Cox regression analysis showed that the mutated MMR gene (i.e., *MSH2* or *MLH1*) and gender were not significant predictors of AO (covariate "G" for MMR gene, hazard ratio 0.952 [95% confidence interval 0.735–1.233], $p = 0.711$; covariate "S" for gender, hazard ratio 0.863 [95% confidence interval 0.664–1.123], $p = 0.274$). However, the genotype of Arg462Gln variant of *RNASEL* was a significant predictor of AO (covariate "R_A", hazard ratio 1.855 [95% confidence interval 1.133–3.038], $p = 0.014$) as well as the genotype of Arg72Pro variant of *p53* (covariate "P_A", hazard ratio 2.247 [95% confidence interval 1.166–4.328], $p = 0.016$), whilst no significant deviation from additivity was noted in both genes (covariate "R_D" for *RNASEL*, hazard ratio 0.883 [95% confidence interval 0.674–1.157], $p = 0.368$, and covariate "P_D" for *p53*, hazard ratio 0.911 [95% confidence interval 0.647–1.282], $p = 0.592$, respectively). Thus, the sequence

Table 4
Distribution of the combination of *p53* Arg72Pro and *RNASEL* Arg462Gln sequence variant among cases and controls and median age of disease onset of the patients with range and 95% confidence intervals

Genotype		Controls ($n = 245$)	Patients ($n = 246$)	Median AO
p53	*RNASEL*	*n* (%)	*n* (%)	years (range; 95% CI)
Arg/Arg	Arg/Arg	49 (20.0)	60 (24.4)	42 (22–75; 39–45)
Arg/Arg	Arg/Gln	76 (31.0)	74 (30.1)	37 (13–69; 34–40)
Arg/Arg	Gln/Gln	25 (10.2)	18 (7.3)	36 (21–48; 32–40)
Arg/Pro	Arg/Arg	35 (14.3)	25 (10.2)	39 (17–59; 32–46)
Arg/Pro	Arg/Gln	35 (14.3)	41 (16.6)	35 (18–67; 32–38)
Arg/Pro	Gln/Gln	8 (3.2)	11 (4.5)	32 (20–49; 29–35)
Pro/Pro	Arg/Arg	3 (1.2)	5 (2.0)	32 (27–34; 21–43)
Pro/Pro	Arg/Gln	11 (4.5)	8 (3.3)	33 (26–48; 21–45)
Pro/Pro	Gln/Gln	3 (1.3)	4 (1.6)	30 (26–47; 23–37)

AO, median age of onset of colorectal cancer in patients (years); CI, confidence interval.

variants in both genes were significantly associated with AO in a dose-dependent manner. The effect of covariate "R_AP_A" which indicates a possible interaction between the two genes, was not significant (hazard ratio 0.770 [95% confidence interval 0.566–1.047], $p = 0.095$), thus suggesting that the combined effect of both variants is purely additive, without interaction.

4. Discussion

We hereby show the influence of two common, functionally different sequence variants (i.e., Arg72-Pro in the tumour suppressor gene *p53* and Arg462Gln in the prostate cancer susceptibility gene *RNASEL*) on the age of onset of colorectal cancer in HNPCC patients who carry pathogenic *MSH2* or *MLH1* germline mutations, demonstrating that there is a purely additive effect between these variants. Previously, we have shown in two independent studies that both variants are significantly associated with AO in HNPCC patients in a dose-dependent manner [16,28].

The AO is not only of clinical importance *per se*, but also because young AO is associated with a poorer 5-year survival in patients with highly microsatellite-unstable colorectal cancers [37]. There are only few other modifying genetic factors reported in association with AO in HNPCC, such as the mutant status of *NAT2*7* [38], and a common variant (c.G870A) in the *cyclin D1* gene [39]; yet, these data could not be corroborated by our findings and those of others [40,41].

p53 and *RNASEL* are tumour suppressor genes involved in apoptosis [17–19,21,42,43]. Therefore, we hypothesise that different apoptotic potentials of the *p53* and *RNASEL* variants modify the disease phenotype, in which the wild-types (i.e., Arg72 in *p53* and Arg462 in *RNASEL*) mediate the destruction of emerging tumours more efficiently than the variants. Accordingly, Pro72 in *p53* and Gln462 in *RNASEL* have been shown to mediate a reduced apoptotic capacity or fail to induce apoptosis in response to 2-5A activation, respectively [11,20]. Consequently, more "attempts" may be required for a successful tumour-initiation in carriers of the wild-type alleles than in those carrying the variant alleles. Because patients with HNPCC have a high life-long probability of developing synchronous and/or metachronous tumours, or both [4], more abortive tumour-initiating events would result in a later clinical manifestation [16,28].

We did not record any significant differences in genotype frequencies between patients and controls, neither in the *p53* Arg72Pro variant nor in the *RNASEL* Arg462Gln variant nor in the combination of both genotypes, suggesting that both variants have no role in tumour initiation. However, we noted a significant association between genotypes and AO for each variant alone as well as for the combination of both genotypes. Whereas the differences in median AO between homozygous carriers of the wild-type and homozygous carriers of the variant allele in each gene alone was six years, the difference in median AO was 12 years between homozygotes for the wild-types and homozygotes for the variant alleles in both genes.

Although both genes (i.e., *p53* and *RNASEL*) act in independent pathways and are tumour suppressor genes inducing apoptosis, a mutual interaction between both genes has not been demonstrated in

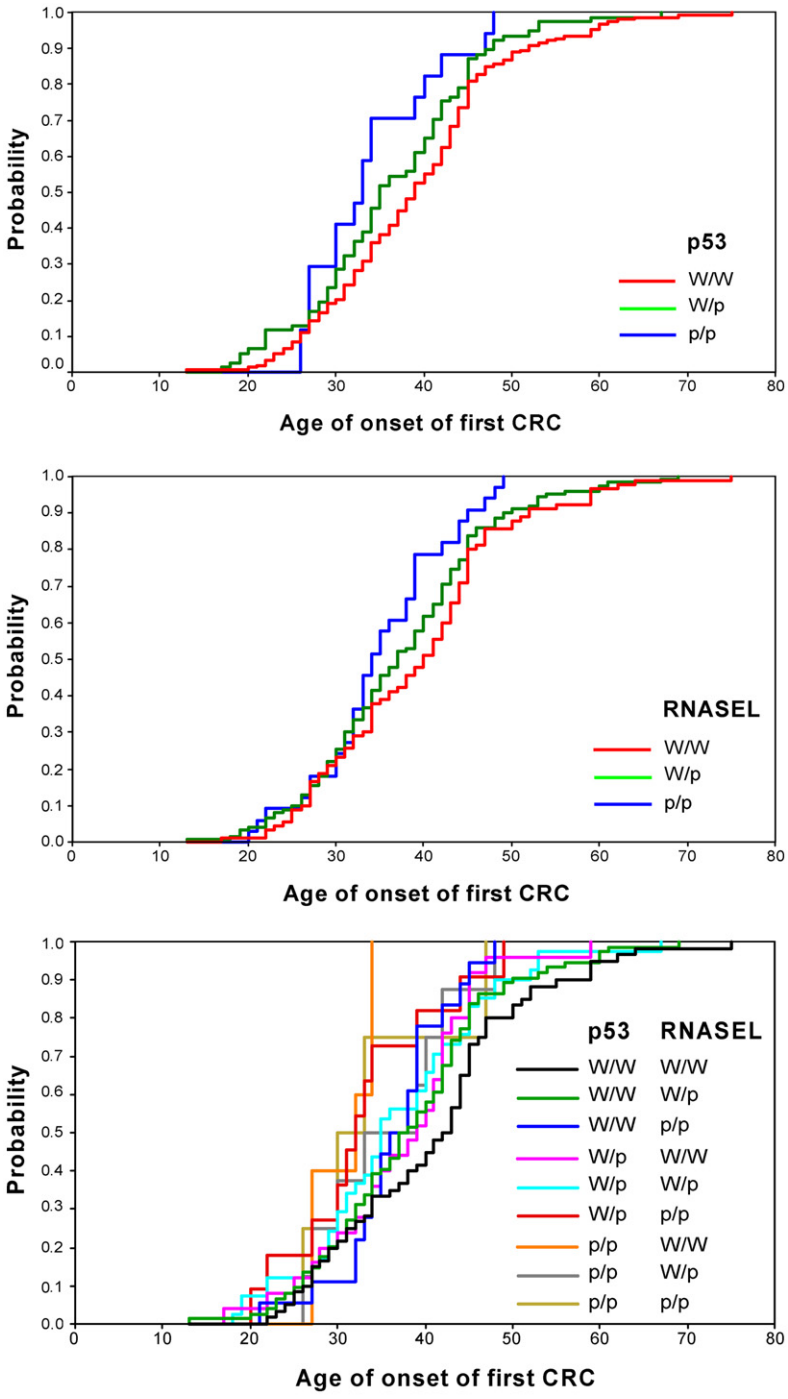

Fig. 1. Age of onset of the first CRC in patients with pathogenic MMR germline mutations and different genotypes of the *p53* c.215G>C (p.Arg72Pro) and *RNASEL* c.1385G>A (p.Arg462Gln) sequence variant ($n = 246$). Above, Kaplan–Meier analysis with genotypes of the *p53* Arg72Pro sequence variant as grouping variable: The AO was significantly different among the three genotype groups in the global comparison ($p = 0.0176$, log rank). Middle: Kaplan–Meier analysis with genotypes of the *RNASEL* Arg462Gln sequence variant as grouping variable – the AO was significantly different among the three genotype groups in the global comparison ($p = 0.0358$, log rank). Bottom: Kaplan–Meier analysis with combinations of genotypes of the *p53* Arg72Pro and *RNASEL* Arg462Gln sequence variant as grouping variable – The age of onset was significantly different among the nine combinations in the global comparison ($p = 0.0174$, log rank). W, wild-type (i.e., *p53* Arg72, *RNASEL* Arg462); p, polymorphic variant (i.e., *p53* Pro72, *RNASEL* Gln462).

an experimental setting. Applying the multivariate Cox regression model, we show that the effect of the variants in both genes is purely additive without interaction, suggesting that there is no interaction between the p53- and Rnase L-pathways.

In conclusion, we report the combined additive effect of two modifying genes on the AO of the phenotype caused by pathogenic germline mutations in *MSH2* or *MHL1* in HNPCC patients. *p53* Arg72Gln and *RNASEL* Arg462Gln sequence variants, together with other modifying factors and additional exogenous and genetic factors may contribute to a more detailed tumour risk assessment in MMR gene mutation carriers. The knowledge of the age of onset of disease in individual carriers of pathogenic MMR germline mutations may have an impact on preventive strategies, including the age of first surveillance, surveillance intervals, and age of preventive surgery.

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Appendix A.

The German HNPCC-Consortium consists of the following centres (in alphabetic order): clinical centres in Bochum (in addition to author: J.T. Epplen, S. Hahn, E. Kunstmann, W. Schmiegel, K. Schulmann, S. Stemmler, J. Willert), Bonn (in addition to authors: R. Büttner, W. Friedl, N. Friedrichs, A. Hirner, C. Lamberti, P. Propping, T. Sauerbruch), Duesseldorf (in addition to author: T.O. Goecke, A. Hansmann, G. Moeslein, C. Poremba, A. Unger, T. Vogel, C. Wieland), Dresden (in addition to authors: D.E. Aust, F. Balck, R. Höhl, F.R. Kreuz, S.R. Pistorius, J. Plaschke, H.D. Saeger), Heidelberg (in addition to author: A. Buckowitz, J. Gebert, P. Kienle, M. Kloor, H.P. Knaebel, U. Mazitschek, M. Tariverdian), Munich–Regensburg (in addition to authors: W. Dietmaier, M. Grabowski, M. Gross, G. Keller, R. Kopp, P. Rümmele, C. Tympner, H. Vogelsang), centre for reference pathology Kassel (in addition to author: T. Brodegger, J. Rüschoff) and centre for documentation and biometry in Leipzig (in addition to author: J. Forberg, M. Herold, M. Löffler).

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