The p53 codon 72 variation is associated with the age of onset of hereditary non-polyposis colorectal cancer (HNPCC)

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The polymorphic variants at codon 72 of the p53 gene were shown to be functionally distinct in vitro, whereby the arginine (arg) variant induces apoptosis more efficiently than the proline (pro) variant. From the evidence that the DNA mismatch repair system and p53 interact to maintain genomic integrity, we hypothesized that the codon 72 variation may influence the age of onset of disease in HNPCC patients. We tested 538 patients for p53 codon 72 variants, including 167 unrelated patients with pathogenic germline mutations in MSH2 or MLH1 and colorectal carcinoma as first tumour, 126 patients with sporadic microsatellite stable colorectal cancers, and 245 healthy controls. The median age of onset was 41, 36, and 32 years for MSH2 or MLH1 mutation carriers with arg/arg, arg/pro, and pro/pro genotypes, respectively. The log-rank test revealed significant differences in the age of onset between arg/arg and pro/pro individuals (p = 0.0002) and in arg/ pro versus arg/arg and pro/pro individuals (p = 0.0026 and p = 0.0217, respectively). A Cox regression model indicated an additive mode of inheritance. No significant differences in age of onset were observed among different genotype carriers with microsatellite stable tumours. Our results suggest that p53 codon 72 genotypes are associated with the age of onset of colorectal carcinoma in a mismatch repair deficient background in a dose dependent manner. These findings may be relevant for preventive strategies in HNPCC.

Several studies have addressed the issue of whether this polymorphism is involved in the development of cancer. As the arg72 and pro72 variants differ in their susceptibility to degradation by human papilloma virus (HPV) E6 protein, the association between these variants and cancer risk has been studied in cervical cancer and in several other types of tumour, with controversial results. Recently, it has been shown that the polymorphic variants at codon 72 of p53 are functionally distinct in vitro, whereby the arg72 variant induces apoptosis more efficiently than the pro72 variant. The data suggest that at least one source of this apoptotic potential is the greater ability of the arg72 variant to localise to the mitochondria, and thus to be associated with p53 dependent apoptosis.

Hereditary nonpolyposis colorectal cancer (HNPCC) is one of the most common colorectal cancer susceptibility syndromes, with an autosomal dominant mode of inheritance and incomplete penetrance. In the majority of cases, it is caused by germline mutations in the DNA mismatch repair (MMR) genes MSH2, MLH1, MSH6, and PMS2, with most of the mutations occurring in MSH2 and MLH1. Mutation carriers have an increased risk of developing colorectal carcinoma, and extracolonic neoplasias such as endometrial, small bowel, ureter/renal pelvis, stomach, ovary, and hepatobiliary cancer. Therefore, a specific surveillance program for early detection of tumours is recommended for these patients.

A hallmark of HNPCC malignancies is the contraction/expansion of simple DNA sequence motifs, a process termed microsatellite instability (MSI). The Bethesda guidelines, which include family history, and number and age of onset of HNPCC associated tumours, are recommended for the identification of patients with tumours with high MSI (MSI-H). In addition to an incomplete penetrance of about 80% for colorectal cancers and the broad tumour spectrum, a wide variety of age of onset of disease ranging from 16 to 90 years has been described. To date, the only genetic factors that have been reported in association with the age of onset of HNPCC are a common variant in the cyclin D1 gene and the mutant status of NAT2, one of several isozymes of N-acetyltransferase. In contrast to microsatellite instability, which is a feature of malignancies associated with MMR deficiency, most of the sporadic colorectal carcinomas show chromosomal instability frequently associated with loss of p53 and the development of aneuploidy. However, there is also evidence that the MMR system and p53 interact to maintain genomic integrity. For example, in vitro analysis of the MLH1 deficient colorectal cancer cell line HCT-116 indicates that p53 can cooperate with the mismatch repair system in protecting cells from DNA damage. In addition, it has been suggested that MLH1 deficient cells seem to be more dependent on the protective
effect of p53 than MMR proficient HCT-116 cells in which MLH1 function was restored. Similar findings have been reported for the ovarian cancer cell line A2780. Furthermore, studies in knockout mice provided evidence for a cooperation between MMR system and p53 in tumorigenesis. We hypothesised that the arg72/pro72 variation in p53 modaulates the phenotype of neoplasias arising from pathogenic germline mutations in mismatch repair genes. To exclude additional unknown exogenous and genetic factors that might modify penetrance, we studied only patients who had developed colorectal cancer. We show a significant association between the p53 codon 72 variation and the age of onset of first manifestation of colorectal cancer in HNPCC patients, but lack of association in individuals with microsatellite stable tumours. These findings support the notion of a cooperation between p53 and the mismatch repair system in vivo.

PATIENTS AND METHODS

Patients

We studied 167 unrelated HNPCC patients consecutively registered at the clinical centres Bochum, Bonn, Düsseldorf, Dresden, Heidelberg, and Munich Regensburg of the German HNPCC Consortium. Bethesda criteria were applied as inclusion criteria. In addition, four patients with young age of onset of colorectal cancer or with a family history suspicious of HNPCC but who did not meet any of the Bethesda criteria were included. To avoid possible familial or genetic factors inducing a correlation in family members (besides the p53 genotype), we included only one member per family. Of 167 individuals studied (103 men, 64 women), 157 were the probands in their family. In the remaining 10 families, a colorectal cancer was not the first tumour presentation in the nominal proband, which was one of the requirements for inclusion in the study. Patients from these 10 families selected for the study were the earliest onset cases for colorectal cancer in their respective families. In all index patients from whom tumour material was available, microsatellite analyses and, in most cases, immunohistochemistry analyses of mismatch repair protein expression of at least MSH2 and MLH1 were performed. Aberrant findings such as MSI-H or lost or reduced expression of at least one MMR protein identified by immunohistochemistry led to mutation screening. In patients who fulfilled Amsterdam I/II criteria but for whom tumour material was not available, mutation screening for MSH2 and MLH1 was performed without results for MSI or immunohistochemistry. All patients described here were carriers of germline mutations in either MSH2 (87 patients) or MLH1 (80 patients), predicted to be pathogenic because of 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 such mutations.

The first tumour manifestation in all 167 patients was a colorectal carcinoma revealed by medical history and histopathological examination. Median age at diagnosis of colorectal cancer in all patients harbouring a MMR germline mutation was 39 years. Tumours of 142 of the 167 kindreds were analysed and revealed MSI-H. Microsatellites from the other 25 families were not analysed because tumour samples were not available. For MSI analysis, at least five markers according to the reference panel of the International Guidelines for Evaluation of MSI in Colorectal Cancer were applied. MSI-H was considered if at least 30% of markers showed instabilities. Apart from the 167 HNPCC patients, 126 patients with sporadic tumours from Dresden were analysed using 15 microsatellite markers. Microsatellite stability (MSM) was considered if none of the markers showed instability. Age of onset of disease was defined as the time of histological tumour diagnosis. All patients gave written informed consent for study participation. The controls were 245 anonymous healthy blood donors from the Dresden Regional Blood Center.

Methods

Genotyping of p53 codon 72 was performed on genomic DNA isolated from peripheral blood lymphocytes by two independent methods as follows.

(1) PCR-amplification (Tag polymerase, Perkin Elmer Applied Biosystems, Weiterstadt, Germany) of exon 4 of p53 using primers 5′-TGAGGACCTTGTCCTGAC-3′ and 5′-AGAGGAATTCGAAATTCCA-3′, resulting in 412 bp fragments. The PCR-products were digested with the endonuclease BstU1 (restriction site: 5′...CGG...3′, 3′...CGG...5′), which specifically cleaves the allele coding for arg72 (CGG) but not that for pro72 (CCC). Cleaved PCR products resulted in two fragments of 161 bp and 251 bp. Fragments were analysed on an agarose gel.

(2) Most of the genotypes were confirmed by single base sequencing of PCR products using the Thermo Sequenase Fluorescent Cycle Sequencing kit and Automated Laser Fluorescence (ALF express) sequencing devices (both Amersham Pharmacia Biotech, Freiburg, Germany). Sequencing was performed with the Cy5 labelled antisense primer 5′-ATACGCGAGCCTGTCAT-3′, using a kit that included cytosine, guanine, or both as dideoxynucleotide. To exclude the occurrence of a third variant, TGC which codes for cysteine, all samples with at least one nondigested allele were screened by single base sequencing using the dideoxynucleotide adenine.

Statistical analysis

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. Firstly, a global comparison of all three groups was performed. Pairwise post hoc comparisons between the genotype groups were only performed after the global test revealed a significant difference. Multivariate Cox regression analysis was used to evaluate the role of the location of the MMR gene defect (MSH2 or MLH1) and to identify the possible mode of inheritance (additive versus dominant). The χ² test was used to evaluate the homogeneity of genotype frequency distributions among the three groups of individuals. Genotype frequencies in all groups were cross checked with Hardy-Weinberg expectations by the χ² test. Significance was set at p<0.05. The statistical software package SPSS was used for all statistical data analyses.

RESULTS

Overall we found 330 arg/arg (61.3%), 173 arg/pro (32.2%) and 35 pro/pro (6.5%) genotypes. The compiled data analysis of the patients and controls is summarised in table 1, and the distribution of genotypes in the two patient groups and in the control group are given in table 2. We did not observe a significant difference in frequency of genotypes among these groups. Genotype frequencies in all groups were in accordance with those previously reported in Europe. No significant deviations from Hardy-Weinberg equilibrium were detected (data not shown).

In patients with MMR germline mutations the median age of onset was 41 years for the arg/arg, 36 years for the arg/pro, and 32 years for the pro/pro individuals (table 3). The age of onset was significantly different between the three genotypes in the global comparison (log rank, p<0.0001). All pairwise
The p53 codon 72 variation is associated with the age of onset of hereditary non-polyposis colorectal cancer (HNPCC) 771

post hoc comparisons between the genotype groups revealed significant differences (arg/arg v pro/pro p = 0.0002, arg/arg v arg/pro p = 0.0026, arg/pro v pro/pro p = 0.0217; see fig 1B).

We used a multivariate Cox regression analysis to investigate whether the age of onset depended on the location of the MMR gene defect (MLH1 versus MSH2) and whether the data could be explained assuming an additive mode of inheritance. The hazard function in this model was defined as $h(t) = h_0(t) \exp(\beta_1 + \beta_2 + \beta_3)$, where $G$ represents an indicator variable for the location of the MMR gene defect (coded as 1 for MLH1 and 2 for MSH2), $A$ represents the number of pro alleles (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, and $D$ measures departure from additivity (that is, dominance, coded as 1 for the arg/pro genotype and 0 for the arg/arg and pro/pro genotypes). The result of the regression analysis showed that the location of the MMR gene defect was not a significant predictor ($\beta_1 = -0.151, p = 0.342$). The effect of covariate A was significant ($\beta_2 = -0.609, p<0.001$); however, no significant deviation from additivity could be found ($\beta_3 = -0.108, p = 0.604$).

In contrast, no significant differences in the age of onset among the p53 genotype carriers were observed in the patients with sporadic microsatellite stable colorectal cancers (log rank, $p = 0.2096$). The median age of onset was 65 years in the arg/arg, 62 years in the arg/pro, and 61 years in the pro/pro individuals (table 3).

DISCUSSION

We observed a significant association between p53 codon 72 variants and age of onset of colorectal cancers in patients with MSH2 and MLH1 germline mutations in a dose dependent manner, but not in patients with microsatellite stable tumours. Notably, a cooperation between MMR system and p53 in tumorigenesis has been reported in knockout mice. Animals nullizygous for both Msh2 and p53 (Msh2−/−, p53−/−) had a significantly reduced median survival time compared with Msh2 deficient animals (Msh2−/−, p53+/+).30,31 Furthermore, Toft et al found a significantly reduced survival of Msh2 deficient mice heterozygous for p53 (Msh2−/−, p53+/−) compared with Msh2 deficient mice proficient for p53 (Msh2−/−, p53+/+) and suggested a dose sensitive role of p53 in the maintenance of genomic integrity at the nucleotide level.31 Notably, young age of onset is associated with a poorer 5 year survival rate in patients with highly unstable colorectal cancers.30

Very recently, another study with a different design but similar results was published by others.37 In contrast to our

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**Table 1** Compiled data analysis of the patients and controls

<table>
<thead>
<tr>
<th>MMR mutation carrier</th>
<th>Sporadic MSS</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>167</td>
<td>126</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>103</td>
<td>74</td>
</tr>
<tr>
<td>Female</td>
<td>64</td>
<td>52</td>
</tr>
<tr>
<td>MMR gene mutated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSH2</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>MLH1</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Median age, in years, of onset of CRC (range)</td>
<td>39 (13–62)</td>
<td>64 (50–94)</td>
</tr>
</tbody>
</table>

*Age: Beth, Bethesda

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**Table 2** Genotype frequencies in all patient groups and controls (CRC = colorectal cancer)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>All genotypes</th>
<th>Healthy controls</th>
<th>Sporadic MSS CRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>arg/pro</td>
<td>538</td>
<td>35</td>
<td>6.5</td>
</tr>
<tr>
<td>arg/arg</td>
<td>245</td>
<td>17</td>
<td>7.0</td>
</tr>
<tr>
<td>arg/pro</td>
<td>126</td>
<td>6</td>
<td>4.8</td>
</tr>
<tr>
<td>arg/arg</td>
<td>167</td>
<td>12</td>
<td>7.2</td>
</tr>
</tbody>
</table>

**Table 3** Age of onset of colorectal cancer (in years)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>pro/pro</th>
<th>arg/arg</th>
<th>arg/pro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporadic MSS, median age</td>
<td>61</td>
<td>62</td>
<td>65</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(54 to 68)</td>
<td>(59 to 65)</td>
<td>(62 to 68)</td>
</tr>
<tr>
<td>MLH1 mutation carrier, median age</td>
<td>32</td>
<td>36</td>
<td>41</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(29 to 35)</td>
<td>(33 to 39)</td>
<td>(39 to 43)</td>
</tr>
</tbody>
</table>

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**Figure 1** Age of onset of the first CRC in patients with pathogenic MMR germline mutations and different genotypes of the p53 codon 72 variants (n = 167) (A) Kaplan-Meier analysis with the location of the MMR gene defect as grouping variable. Cox regression analysis did not reveal a significant influence of the location of the MMR gene defect on age of onset. (B) Kaplan-Meier analysis with genotypes of the p53 codon 72 variants as grouping variable. The age of onset was significantly different among the three genotype groups (global p<0.0001) and in all pairwise comparisons (arg/arg v pro/pro p = 0.0002, arg/arg v arg/pro p = 0.0026, arg/pro v pro/pro p = 0.0217). Cox regression analysis indicated an additive mode of inheritance.
study, 92 MSH2 or MLH1 mutation carriers from 47 families were included, and 16 families were represented by more than one member (range 2–8). In addition to 62 subjects with truncation or deletion mutations, 30 (32.6%) missense mutation carriers were also analysed. Of the 92 individuals studied, 47 had colorectal cancer and 45 (48.9%) were unaffected MMR mutation carriers. As with our study, the authors found a significantly earlier median age of onset in individuals who were heterozygous (arg/pro) than in those who were homozygous for the wild type (arg/arg) p53 allele. Only seven subjects were homozygous for the polymorphic p53 alleles (pro/pro) and only one of these had colorectal cancer (at 63 years of age). According to the authors, this number was too small to provide meaningful results. For this reason, a dose dependent effect of the variant p53 allele could not be shown.

We did not observe significant differences in genotype frequencies between the two groups of colorectal cancer patients and controls, suggesting that the p53 codon 72 variation is not involved in tumour initiation. This finding is in accordance with several studies that failed to detect an association between the occurrence of malignancies and the p53 codon 72 variation in various tumour types including colorectal cancers, although others reported quite opposite results.

One substantial difference between the experiments in mice and our clinical study is that although both approaches used an MMR deficient background, the animal experiments studied inactivating mutations of p53, while we analysed p53 variants differing in their potential to induce apoptosis. Notably, we still observed a linear trend in the age of onset across genotype groups in colorectal cancer patients with MMR deficiency. These findings suggest that the mode of action of p53 in a mismatch repair deficient background is the induction of apoptosis in developing tumours.

We therefore postulate that the different apoptotic potentials of the p53 codon 72 variants modify the disease phenotype, and that the high apoptosis variant arg72 mediates the destruction of MSI-H tumours more efficiently than the low apoptosis variant pro72. There is a high probability in HNPCC patients of developing synchronous and/or metachronous tumours during their lifetime. However, more “attempts” to develop a tumour may be required in carriers of the high apoptosis p53 variant than in those who carry the low apoptosis variant. Given the almost linear risk increase of developing an HNPCC tumour during a lifetime, the more “attempts” needed to develop a tumour, the later the clinical manifestation.

As p53 is involved in numerous cellular pathways related to carcinogenesis through multiple, complex interactions that are only partially understood, other possible mechanisms should be considered. For example, another explanation for the observed association between the p53 codon 72 variants and age of onset in patients with MSI-H tumours, but not in patients with MSS tumours, could be that microsatellite stable colorectal cancers more frequently exhibit somatic alterations in p53 with loss of function than highly microsatellite unstable cancers. Thus, inherited variants in p53, such as the codon 72 polymorphism, would have a minor impact in patients with MSS tumours. MMR insufficient cells may also be more dependent on the p53 mediated apoptotic pathway, as the MMR system itself seems to play a potential role in apoptosis in a largely p53 independent manner. In addition, a dose sensitive role for p53, resulting in suppression of Msi1 in the absence of Msh2, has been considered at the nucleotide level in a mouse model. Furthermore, p53 deficient cells are incapable of damage induced G1/S checkpoint arrest, which may result in slower tumour growth.

If independently corroborated by prospective cohort studies, the observed effect of the p53 codon 72 variation on the age of onset in HNPCC should be robust in populations with different allele frequencies in various regions of the northern hemisphere. Similarly, it can also be inferred that the median age of onset of HNPCC in populations with a high allele frequency of the pro72 variant such as Nigerians or African Americans will be lower than in white populations.

In conclusion, our data support the notion that colorectal cancer is a multifactorial disease. p53 codon 72 variants, in conjunction with other modifying factors such as cyclin D1 polymorphism, MMR, 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 germine mutations may have an impact on preventive strategies, including the age at first surveillance, surveillance intervals, and age at preventive surgery.

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