Polymorphism of the p73 gene in relation to colorectal cancer risk and survival

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The results regarding a GC/AT polymorphism in the p73 gene in relation to cancer risk are inconsistent, and the significance of loss of heterozygosity (LOH) of the gene is unclear. In the present study, we investigated whether this polymorphism was related to the risk of colorectal cancer, and whether there were relationships between the polymorphism and LOH, protein expression or clinicopathological variables. 179 patients with colorectal cancer and 260 healthy controls were genotyped for the polymorphism by PCR-restriction fragment length polymorphism (RFLP). Fifty informative cases were examined for LOH in tumours. Immunohistochemistry was performed on distant (n = 42) and adjacent normal mucosa (n = 33), primary tumour (n = 69) and lymph node metastasis (n = 12). The frequencies of the genotypes were 63% for wild-type (GC/GC), 30% for heterozygotes (GC/AT) and 7% for variants (AT/AT) in patients, and 62, 36 and 2% in controls, respectively. The frequencies of the genotypes in the patients and controls were significantly different (P = 0.02). The patients carrying the AT allele had a better prognosis than those with the GC/GC genotype (OR = 0.42, 95% CI = 1.15-5.02, P = 0.02). No LOH was observed at the p73 locus. Expression of p73 protein was increased from normal mucosa to primary tumours (P = 0.02), but was not significantly changed between primary tumours and metastases (P = 1.0). In conclusion, the AT/AT homozygotes may have a greater risk of developing colorectal cancer, while the patients who carried the AT allele had a better prognosis.

Introduction

The p73 gene has been located to the 1p36 locus, a site observed to have frequent loss of heterozygosity in different types of cancers (1–3). The p73 protein has a structural and functional homology with p53, a known tumour suppressor (1). Therefore, it was proposed that p73 was the long sought tumour suppressor candidate (4). However, accumulating evidence has shown that neither complete loss nor any significant mutation of the p73 gene in many types of tumours is commonly occurring. Recently, Bénard et al. reviewed 1426 various human tumours, and showed that only 0.6% of the cases had missense mutations and 20% had LOH of the p73 (2). The

mutations found had no significant effect on the transcriptional activity and growth-suppressing ability (2). It has also been observed that p73 null mice do not show any spontaneous tumour development although they have a high mortality, mostly caused by massive haemorrhages in the gastrointestinal tract (5). Therefore, p73 is now included as a classic tumour suppressor (1,2). Over-expression of the p73 protein has been seen in benign and malignant tumours including colorectal cancer, when compared with the matched normal tissues (6,7). Furthermore, p73 over-expression was correlated with a poor prognosis in colorectal, hepatoacellular and breast cancers (2,6).

The TP73 gene contains a polymorphism just upstream of the initiating AUG of exon 2, a region that theoretically might form a stem-loop structure and has been thought to affect p73 function by altering gene expression. It is a linked polymorphism formed by two single polymorphisms at positions 4 (G→A) and 14 (C→T). Since they are in complete linkage equilibrium, only three genotypes, GC/GC, GC/AT and AT/AT, are available (3,8–11). Hamajima et al. performed a study of the GC/AT polymorphism correlated to the risk of oesophageal, stomach and colorectal cancers in a Japanese population, but they found no significant correlation (11). Ryan et al. found that AT/AT homozygotes were significantly less prevalent in the oesophageal cancer patients, compared with the controls in an Irish population. They also observed 37.8% of LOH in the heterozygote patients (8). In contrast, very recently, Li et al. indicated that AT allele and genotypes were significantly related to risk of squamous cell carcinoma of the head and neck in an American population (10).

In the present study, we investigated whether this polymorphism was related to the risk of colorectal cancer in a Swedish population, and whether there were relationships between the polymorphism and LOH, protein expression or clinicopathological variables.

Materials and methods

Materials

In the case-control study using the PCR-restriction fragment length polymorphism (RFLP) assay, case DNA used was from the normal mucosa of 179 patients, and for LOH analysis, DNA was derived from 50 tumours, whose matched DNA from normal mucosa was shown to be heterozygous. For immunohistochemistry, the staining was performed on distant (n = 42) and adjacent (n = 33) normal mucosa, primary tumour (n = 69) and lymph node metastasis (n = 12). The distant normal mucosa samples were taken from the margin of distant resection, and were histologically free of pre-cancer and cancer. All patients were diagnosed at the Department of Pathology, Linköping and Vrinnevi Hospitals, Sweden. The patients’ gender, age, tumour location and Dukes’ stage were obtained from surgical and pathological records. There were 96 males and 83 females. The mean age was 71 years (ranging from 35 to 95 years). The growth pattern was based on the patterns of growth and invasiveness. Differentiation was graded as well, moderately or poorly differentiated. Fifteen patients had received radiotherapy, 115 had not, and 49 had no available information. Eighteen patients had received chemotherapy, 112 had not, and 49 had no information available. Eight patients had received both radiotherapy and chemotherapy. The patients were followed-up until the end of

Abbreviation: RFLP, restriction fragment length polymorphism.
2003. By that time, 54 patients had died from colorectal cancer. There was no information available on tumour location in three cases, Dukes’ stage in 12, growth pattern in 87 and differentiation in five.

Controls comprised 260 healthy blood donors, who were in the same residential area as the patients. The individuals had neither gastrointestinal diseases nor a history of tumours. Among controls, 126 were male and 134 were female, and the mean age was 54 and median was 59 years (ranging from 22 to 77 years).

A melanoma cell line was used for testing the specificity of the p73 antibody.

DNA extraction
The genomic DNA was extracted from 20 mg distant normal colorectal mucosa (n = 179) and colorectal tumour tissue (n = 50), respectively, by means of the Wizard® SV Genomic DNA Purification System according to the manufacturer’s instructions (Promega, Madison, WI). The concentration and purity of the DNA were measured with a spectrophotometer. The genomic DNA from the controls was extracted from peripheral leukocytes also by means of the Wizard® Genomic Purification Kit (Promega).

PCR–RFLP
PCR was performed in a 20 µl reaction mixture containing ~25 ng chromosomal DNA, 1 × PCR buffer (Promega), 1.5 mM MgCl2 (Promega), 0.2 mM dNTP, 0.5 mM of each primer (SGS, Køge, Denmark) and H2O. The primer sequences were 5′-CGAGAG-ACGAGGCAGGAGGCAGGAGG-3′ and 5′-CGAGGATGCGAGGCTGAG-3′. The PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s and a final extension for 10 min at 72°C. The 229 bp PCR-products were checked on an agarose gel, stained with ethidium bromide, in UV-light.

The PCR products were digested by the restriction enzyme Sryl (Fermentas, Vilnius, Lithuania), which had a digestion site when the AT allele was present. Cleaving of the AT allele yielded two products, 157 and 72 bp, respectively. The wild-type, GC allele, remained uncleaved by Sryl, yielding a 229 bp product. The products were resolved on a 3% agarose gel.

Loss of heterozygosity
PCR on DNA from tumours and the matched DNA from normal mucosa (that was heterozygous for the polymorphism) was performed at the same PCR run, using the same protocol as mentioned above. The PCR products were cleaved by Sryl (Fermentas), and checked on a 3% agarose gel. The genotype of the tumours was compared with that of the matched normal mucosa side-by-side on the same gel. If the three bands (72, 157 and 229 bp) were visible, the cases were considered as heterozygotes. We repeated the experiments at least two times, including PCR and enzyme cleavage, to ensure the results, and the data were consistent.

Western blotting
The p73 antibody used was an affinity-purified goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) against a peptide in the C-terminus of the human isoform p73b. The antibody reacts with both p73a and p73b of human origin. The proteins from the melanoma cell lines were separated through electrophoresis at 200 V for 1 h and then transferred from the electrophoresis gel to a nitrocellulose membrane at 100 V for another 1 h. The membrane was washed in PBS-Tween three times and incubated with blocking milk for 2 h. After another PBS-wash the membrane was incubated with the primary antibody at 4°C over night. After washing the membrane it was incubated with a secondary antibody (DAKO, Carpilenta, CA) for 2 h. Finally, after washing, an ECL Plus reagent was added and the membrane was then developed by exposure onto film, showing only one band at 73 kDa.

Immunohistochemistry
Sections from paraffin-embedded tissue blocks were incubated in an oven at 60°C for 12 h, and were then deparaffinized and hydrated in descending concentrations of ethanol and finally in double distilled H2O. To expose masked epitopes, the sections were microwaved in citrate buffer (pH 6.0) for 2 × 5 min, and then kept at room temperature for 30 min, followed by a PBS-wash for 2 min. The activity of endogenous peroxidase was blocked in 3% H2O2 in methanol, and then the sections were washed three times in PBS. After blocking with 1.5% blocking serum in PBS (goat ABC Staining System, sc-2023, Santa Cruz Biotechnology) for 10 min, the primary antibody (Santa Cruz Biotechnology) was added at 1:150 in antibody diluent (DAKO) and then incubated at 4°C over night. After washing with PBS, a biotinylated secondary antibody (ABC Staining System, Santa Cruz Biotechnology) was added. After 30 min the sections were rinsed with PBS. AB enzyme reagent (ABC Staining System, Santa Cruz Biotechnology), containing avidin, biotinylated horse-radish peroxidase and PBS, was then added to the slides. The AB enzyme reagent was rinsed off after another 30 min with PBS. Peroxidase substrate (ABC Staining System, Santa Cruz Biotechnology) containing 3,3'-diaminobenzidine chromogene, peroxidase substrate, buffer and distilled H2O, was added and incubated for 10 min and then rinsed with water. The sections were then counterstained with haematoxylin. In all runs, negative controls were included, where PBS was used instead of the primary antibody, and neither showed staining.

Two independent investigators, without any knowledge of the clinicopathological data, graded the slides in a blinded fashion. The cases were graded as negative, weak, moderate or strong, based on the intensity of the staining. The staining patterns were graded as cytoplasmic, nuclear or both. The percentage of the staining was graded as <5, <25, <50, <75 and >75%. Finally, the staining intensity was closely compared between the available samples of distant, adjacent normal mucosa, primary tumour, and metastasis from the same patient. In the cases with discrepant scoring, a consensus score was reached after re-examination. To avoid artificial effect, cells in areas with necrosis, poor morphology or in the margins of sections were not counted.

Statistical analysis
The χ² method was used to test the frequencies of genotypes/alleles in colorectal cancer patients with the control population or clinicopathological variables. McNemar’s method was used for testing differences of the genotype/allele frequency in normal colorectal mucosa and tumour. Logistic regression was used for calculating odds ratio (OR), 95% confidence intervals (CI) and trend test. Cox’s Proportional Hazard Model was used to test the relationship between the polymorphisms and the survival of patients. Survival curves were computed according to the Kaplan-Meier method. All P-values cited were two-sided and P-values <0.05 were judged as statistically significant.

Results
The p73 polymorphism in colorectal cancer patients and healthy controls
The frequencies of the three genotypes, GC/GC, GC/AT and AT/AT, between the patients and controls were significantly different (P = 0.02, Table I). Apparently, the patients had a higher frequency of AT/AT (7 versus 2%) but a lower frequency of GC/AT genotype (30 versus 37%), compared with that of the controls (P = 0.61 for a trend test). There was no statistically significant difference in the allele frequency between cases and controls (P = 0.61, data not shown). We did not observe an association of this polymorphism with age in the control group, with cut-off points of <50, 50-69 and ≥70 years old (P = 0.14, data not shown).

A significant correlation between the genotypes and survival was found in the patients (P = 0.01, Figure 1). Furthermore, the patients with the GC/GC genotype had a worse prognosis compared with those with both AT/AT and GC/AT (OR = 0.42, 95% CI = 1.15-5.02, P = 0.02). We did not find any relationship of the genotypes with gender, age, tumour site, Dukes’ stage, growth patterns and differentiation (P > 0.05, Table II), neither with chemotherapy and/or radiotherapy (P > 0.05).

Matched tumour tissue was available for 50 of the 54 heterozygotic cases, and did not show LOH of the p73 gene (data not shown).

### Table I. Frequencies of p73 genotype in colorectal cancer patients and healthy controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients</th>
<th>Controls</th>
<th>OR (95% CI)</th>
<th>Age adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC/GC</td>
<td>113 (63%)</td>
<td>159 (61%)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>GC/AT</td>
<td>54 (30%)</td>
<td>96 (37%)</td>
<td>0.79 (0.53-1.19)</td>
<td></td>
</tr>
<tr>
<td>AT/AT</td>
<td>12 (7%)</td>
<td>5 (2%)</td>
<td>3.38 (1.16-9.85)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P = 0.02</td>
<td>P = 0.12</td>
</tr>
</tbody>
</table>

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The relationship between the p73 polymorphism and immunohistochemical staining

The genotypes of the polymorphism and the staining intensity in normal mucosa (including distant and adjacent normal mucosa) and tumours were analysed. It seemed that, tumours with the AT/AT genotype had a tendency to be less stained while normal samples did not show such a trend (Table III). We did not do statistical analyses since there were too few cases.

The staining intensity and the percentages of stained cells were also compared. The results showed that, in a majority of the cases, when the staining intensity was strong the percentage of stained cells was also high and vice versa (data not shown).

Discussion

In the present study, we found a relationship of the AT/AT polymorphism with a high risk of colorectal cancer development in a Swedish population. Hamajima et al. did not find any relationships between this polymorphism and the risk of the digestive tract cancers including colorectal cancers in a Japanese population (11). Li et al. indicated that the AT allele and genotypes were related to risk of head and neck cancers in an American population (10), but Ryan et al. had an opposite result in esophageal cancers in an Irish population (8). The different results pointed towards differences of the genotype frequency between the populations, possibly based on race, gender, age, environmental and social factors, and even clinicopathological characteristics of the patients. Considering the controls in the four populations, GC/GC, AT/GC and AT/AT were 61, 37 and 2% in Swedish, 63, 32 and 6% in American, 47, 43 and 10% in Irish, and 55, 40 and 4% in Japanese. There

Table III. Relationship between p73 polymorphism and staining intensity

<table>
<thead>
<tr>
<th>Staining</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GC/GC</td>
</tr>
<tr>
<td>Normal</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>Strong</td>
</tr>
<tr>
<td>Tumour</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>Strong</td>
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</table>

*For McNemar’s test.

We compared the staining intensity in distant normal mucosa with adjacent normal mucosa, primary tumour or metastasis. There was no significant difference between the distant and the adjacent normal mucosa (P = 0.48). However, the staining intensity of the tumour was significantly increased compared with either distant (P = 0.02, Table IV, Figure 2) or adjacent normal mucosa (P = 0.02). There was no significant difference between primary tumours and metastases (P = 1.0). A further comparison of the staining intensity in 12 patients that had a complete data set of distant and/or adjacent normal mucosa, primary tumour and metastasis. When the tumours were compared with distant and/or adjacent normal mucosa, five had stronger, five had the same and two had a weaker staining. Compared with primary tumours, three metastases showed stronger, six the same and three weaker staining.
were statistical differences of the genotype frequency between Swedish and American \( (P = 0.02) \) or Irish \( (P = 0.0003) \) \( (P = 0.15 \) for Swedish versus Japanese). The differences in the genotype frequency between the Swedish and Irish controls were not explained by the race since the two populations are Caucasian, generally sharing a western lifestyle. However, there might be differences regarding environmental and social factors, such as smoking, intake of alcohol and fat consumption, some known to affect the digestive tract cancer risk. It seems that this polymorphism was not related to age \( (9,12) \). Li et al. did not observe an age-polymorphism association in 1229 healthy controls. It is unlikely that the individual’s gender played a role regarding the differences since the controls (48 versus 49\%) and patients (54 versus 58\%) in Swedish and Japanese had similar distributions of gender (there was no available information for the Irish population). It is understandable though that the polymorphism may play a different role in different types of cancers. Finally, we could not exclude a possibility that the varied results were due to a small number of the cases, especially the AT/AT group, analysed in the studies. Therefore, the evidence of the AT/AT related to the risk of colorectal cancer in this study and of the other types of cancers studied by others needs to be confirmed in extended numbers of cases. In addition, similar to the other case-control studies on the different cancer types \( (11,12) \), we also observed that the patients had a higher frequency of AT/AT (7 versus 2\%, OR = 3.38) but a lower frequency of GC/AT genotype (30 versus 37\%, OR = 0.79), compared with that of the controls. The mechanism for this is unclear.

We found a significant correlation between the genotypes and survival in the present study. The patients with AT/AT and GC/AT genotypes had a more favourable survival than those with the GC/GC genotype. Previous studies have shown that over-expression of the p73 protein is a poor prognostic factor in colorectal cancer patients \( (6,7) \). Here, we further observed that the AT/AT allele showed a slight tendency towards lower expression of the p73 protein in tumour, which might be an explanation for the better survival rate in AT/AT allele carrying patients. A possible explanation for the tendency towards less protein expression with AT/AT homozygotes might be that the change from GC to AT might lead to formation of a stem–loop structure, thus influencing the translation efficiency of p73 in tumours. Our present results further genetically supported the evidence that the over-expression of the p73 protein was related to a poor prognosis in cancer patients. Considering the normal mucosa, where the genotype was not related to the protein expression, it might be that there were many changed biological factors in tumours, but not in normal mucosa, that may affect p73 expression. Unexpectedly, our results taken together, seemed to show that there was a higher risk in developing colorectal cancer if individuals carried the AT/AT genotype, but once affected, the patients had a better prognosis. Notably, in the inherited cancer syndromes, such as hereditary non-polyposis colorectal cancer (HNPCC), the family members with certain germline mutations have a greater risk of developing colorectal cancer, however, affected individuals have a better prognosis than those with sporadic colorectal cancer \( (13) \). Because of the polymorphism being a hereditary factor, the individuals with the polymorphism may share certain biological and clinical features with the patients who have the germline mutations for developing HNPCC.

The polymorphism in the p73 gene is not the only factor involved in colorectal cancer development, but it might act as an enhancer of the progress, since p73 has been seen to interact with many genes involved in cancer development. It has, for example, been seen to interact with E2F-1, which increases the abundance of p73 mRNA. The levels of p73 then fluctuate through the cell cycle as a response to the E2F-1 activity \( (14) \). Further investigation of the mechanisms behind this polymorphism and the functions and distribution of the different isoforms in different types of cancers, may offer further clarification of the role of p73 in colorectal cancer development. Some theories trying to explain the p73 over-expression have been proposed. For example, it was found that p73 was activated by cell starvation, suggesting that the over-expression may be triggered by the physiological stresses such as hypoxia or nutrient deprivation during tumour growth \( (15) \). It would be of interest to see whether any of the isoforms, the pro-apoptotic TAp73 or the anti-apoptotic DNp73, were specifically expressed or if all of the isoforms in a balanced manner cause the over-expression. It has been shown that the ratio between p73\( \alpha \) and p73\( \beta \) was 1.6:1 in both normal and tumour tissue, which means that the over-expression was not due to an imbalance in the processing of at least those two transcripts \( (16) \).

A region with frequent LOH is 1p36 in different types of cancers, although the frequencies reported vary greatly \( (17) \). In addition, the more exact location has not been mapped precisely \( (17) \) and therefore we looked at the possibility that the losses could be found around the GC/AT polymorphism of the p73 gene. This was not the case though since none of the samples that were heterozygous in normal colorectal tissue...
were lost in the matched primary tumours. We did not exclude a possibility that the lack of LOH could be caused by a contamination with DNA from normal cells in the tumour samples, thereby giving a false result in the genotyping. However, the immunohistochemical staining did not support the theory though, since the heterozygotic samples did not express a lower level of the p73 protein comparing with the samples with other genotypes, which could have been expected if a gene was in fact lost. Another theory is that there is LOH in the p73 gene or elsewhere in the 1p36 locus. We only looked at the 229 bp surrounding the polymorphism. Our findings therefore might support that LOH of the p73 was not a frequent event in colorectal cancer, at least not in the area around the polymorphism.

In conclusion, the patients with the AT/AT genotype may have increased risk of developing colorectal cancer. The patients who carried the AT allele had a better prognosis.

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References


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