

***SIGNIFICANCE OF GENETIC INSTABILITY IN
DEVELOPMENT AND CHEMOSENSITIVITY OF
MALIGNANT TUMORS***

Ph.D. Thesis

Judit Olasz

*National Institute of Oncology
Department of Pathogenetics*

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INTRODUCTION

1. Cancer and genetic instability

Cellular responses to DNA damages have a critical role in tumor genesis. These response mechanisms comprise the processes of DNA damage sensation, cell-cycle arrest, DNA-repair and apoptosis. Inherited or acquired alterations in “care taker” genes, which are involved in the maintenance of genomic integrity, may lead to uncontrolled cell proliferation and accumulation of genetic errors.

1.1 Mismatch repair, genetic instability and chemoresistance

The mismatch repair (MMR) system recognizes and repairs misincorporated bases, as well as small insertion or deletion loops arising during DNA replication. MMR components have also been implicated in cell-cycle regulation and the p53-dependent apoptotic response to a variety of DNA damage. These functions on the one hand promote genetic stability, on the other hand are relevant to the chemotherapeutic sensitivity. In human cells, mismatch recognition is performed by hMSH2 heterodimerized either with hMSH6 for base-base mismatches and loops of one or a few nucleotides or with hMSH3 for insertion/deletion of two or more extrahelical bases. These complexes then interact with another heterodimeric complex, composed of hMLH1 and hPMS2.

1.1.1. Mismatch repair defects in colorectal carcinoma

Germline alterations of the MMR genes result in autosomal dominantly inherited predisposition to hereditary nonpolyposis colon carcinoma (HNPCC). This syndrome is characterized by early onset colorectal carcinoma and extracolonic epithelial-derived tumors most often located in the gastrointestinal and the urogenital tracts. In the majority of the HNPCC cases, germline mutations of the hMLH1 and/or hMSH2 genes can be demonstrated. The hallmark of mismatch repair deficiency is microsatellite instability (MSI) that is alteration in length of short repetitive sequences of the genome by small deletions or insertions. Amsterdam criteria (I and II) and Bethesda guidelines serve as bases for patient selection. Silencing of hMLH1 gene by promoter methylation occurs mainly in sporadic tumors and this is the principal mechanism of MMR inactivation in sporadic colorectal cancers with high microsatellite instability. Losses or defects of MMR factors can confer resistance to cisplatin. This resistance could either be explained by secondary mutations in effectors of apoptosis due to genetic instability, or by the failure of MMR in linking the detection of damage to

apoptosis. In addition to the platinum derivatives, loss of function of the MMR is associated with resistance to anthracyclines and fluoropyrimidines.

1.1.2. Chemosensitivity of testicular germ cell tumors and the MMR

The majority of testicular germ cell tumors (TGCTs) are highly sensitive to cisplatin-based chemotherapy, but a small fraction of cases are resistant to the effects of chemotherapy. The exquisite chemosensitivity of GCTs seems to be the consequence of several factors, including the lack of drug export and detoxification mechanisms, low DNA-repair capacity, sensitive DNA-damage detection systems with initiation and execution of apoptotic pathways. For the development of a resistant phenotype no uniform explanation can be offered. A recent study suggested that failure to initiate apoptosis due to defects in MMR might contribute to resistance.

1.2. p53 - the guardian of the genomic integrity

The tumor suppressor protein p53 plays a critical role in the cellular response to DNA damage by regulating genes involved in cell cycle progression, apoptosis and genomic stability. More than half of all human cancers lose the p53 function by mutation. Inactivation of the p53 pathway may lead to the selection of more aggressive tumors with a high degree of genetic instability, which can be associated with poor prognosis. As diverse drugs can kill tumor cells by activating common apoptotic pathways, mutations that disable p53-dependent apoptosis can produce multidrug resistance. The majority of tumor-derived mutations are missense mutations mapped to the central DNA binding domain.

Missense mutations in p53 may result also in dominant negative and oncogenic effects. The dominant negative effect corresponds to the capacity of the mutant protein to complex with the product of the wild-type allele to inactivate its function. P53 mutants can form hetero-tetramers with other members of the p53 family (p63 and p73) leading to inactivation their ability to induce apoptosis. The binding affinity of mutant p53 to p73 is influenced by a common p53 polymorphism at residue 72. p53 mutants with R72 allele proved to be more potent inhibitors of chemotherapy-induced apoptosis than those of P72 variants. The oncogenic effects of mutant p53 may partly be related to that p53 mutants seem to transactivate or repress specific genes (MDR1, c-myc, PCNA, EGFR).

AIMS OF THE THESIS

In this study we examined such genetic events which set a permissive background for the selection of malignant tumor cells, and also have an effect on therapeutic sensitivity. The mismatch repair system and the p53 protein as representatives of “care takers” are involved in the maintenance of the genomic integrity. We set the aim to examine the effects of the main mismatch repair genes (hMLH1 and hMSH2) in the predisposition to hereditary colorectal cancer, and the influence of these genes on the therapeutic sensitivity of testicular germ-cell tumors. We investigated mutations of the p53 gene in primary head and neck tumors with the view to outline their prognostic values in the clinical outcome and the therapeutic responsiveness.

The following issues were set for investigation:

A) Mismatch repair genes and proteins in hereditary nonpolyposis colorectal carcinomas (HNPCCs) and testicular germ cell tumors (TGCTs)

1. The germline mutational spectrum of Hungarian HNPCC families.
 - 1.1. Establishment of the most appropriate method to screen HNPCC suspected patients.
 - 1.2. Definition of germline mutations and polymorphisms in hMLH1 and hMSH2 genes of the selected patients and evaluation of their impact on disease development following pedigree analysis.
2. Evaluation of mismatch repair deficiency, microsatellite instability and hMLH1 methylation as predictive markers in chemotherapeutic sensitivity of TGCTs.

B) Investigation of the p53 gene in head and neck tumors

1. Analysis of prognostic value of p53 mutations in primary head and neck squamous cell carcinomas (HNSCCs) and normal appearing resection margins.
2. The role of p53 R72P polymorphism in the clinical outcome of HNSCC patients.

PRESENTATIONS RELATED TO THE THESIS

Olasz J, Mándoky L, Géczy L, Bodrogi I, Csuka O, Bak M: Mismatch Repair Deficiency in Testicular Germ-cell Tumors. International Seminar on Drug Resistance in Cancer, (SZAB Biológiai Szakbizottság Ülése): Szeged, 5th December 2005.

Olasz J, Tanyi M, Tóth L, Damjanovich L, Spengler G, Csuka O, Bak M: A genetikai polimorfizmusok szerepe a HNPCC korai kialakulásában. Ph.D. Tudományos Nap, Szeged, 2006. május 3.

Olasz J., Miklós T., Kámory E., Tóth L., Damjanovich L., Csuka O.: The role of genetic polymorphisms in the early manifestation of HNPCC. 19th European Association for Cancer Research meeting, 1-4 July, 2006.

Olasz J., Remenár É., Udvaros I., Kásler M., Csuka O.: Fej-nyak daganatok genetikai markereinek vizsgálata. A Magyar Biokémiai Egyesület Molekuláris Biológiai Szakosztályának 4. Munkaértekezlete Eger, 1999. május 10-13.

PUBLICATIONS RELATED TO THE THESIS

Olasz J, Mandoky L, Geczy L, Bodrogi I, Csuka O, Bak M.: Influence of hMLH1 methylation, mismatch repair deficiency and microsatellite instability on chemoresistance of testicular germ-cell tumors. *Anticancer Res.* 25: 4319-24, 2005.

Tanyi M, **Olasz J**, Lukacs G, Csuka O, Toth L, Szentirmay Z, Rész Z, Barta Z, Tanyi JL, Damjanovich L.: Pedigree and genetic analysis of a novel mutation carrier patient suffering from hereditary nonpolyposis colorectal cancer. *World J Gastroenterol.* 12: 1192-7, 2006.

Kámory E, Tanyi M, Kolacsek O, **Olasz J**, Toth L, Damjanovich L, Csuka O: Two germline alterations in mismatch repair genes found in a HNPCC patient with poor family history. *Pathol Oncol Res.* 12: 228-33, 2006.

Tanyi M, **Olasz J**, Kámory E, Csuka O, Tanyi JL, Rész Z, Damjanovich L: Difficulties in recognizing families with Hereditary Non-polyposis Colorectal Carcinoma. Presentation of 4 families with proven mutation accepted in *EJSO*.

PUBLICATION NOT RELATED TO THE THESIS

Olasz J, Juhász A, Remenár É, Engi H, Bak M, Csuka O, Kásler M. RAR β 2 suppression in head and neck squamous cell carcinoma correlates with site, histology and age. *Oncol Rep.* 18: 105-112, 2007.

The analysis of resection margins has revealed that almost the one third of the normal appearing samples harbor the mutation of the primary tumor. Mutational analysis of surgical margins may therefore be of value in the prediction of local recurrence and in the decision making process for postoperative therapy.

Analyzing the R72P polymorphism of p53 gene in HNSCC patients we concluded that the codon 72 genotype alone has no predictive value for clinical outcome, but allelotyping of the mutant allele may be useful to predict therapeutic sensitivity.

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MATERIALS AND METHODS

1. Examination of mismatch repair in HNPCC and TGCT patients

1.1. Patients

1.1.1. HNPCC suspected patients: 36 patients, operated at the 1st Department of Surgery, University of Debrecen, Medical and Health Sciences Center between 2003 and 2005, were selected on the basis of Bethesda Guidelines. Two index patients with very early tumor manifestation and their cooperative family members were involved in pedigree analysis.

1.1.2. TGCT patients: Specimens of 51 patients with TGCT were collected between 1993 and 2003 at the National Institute of Oncology, Budapest. Prior to surgery patients received neither radio- nor chemotherapy. The tumors were histopathologically classified according to the WHO criteria. Staging was based on UICC classification. Early stage was defined as stage I or stage II/A. Late stage was defined as stage II/B, II/C or stage III. Patients were considered refractory when progression or relapse occurred despite of adequate initial or salvage treatment. Patients with a complete remission and relapse-free follow-up of more than one year were considered as chemosensitive.

1.2. Immunohistochemistry

Paraffin embedded tissue sections of HNPCC and TGCT samples were deparaffinized. Following heat induced epitope retrieval immunohistochemistry was performed using mouse anti hMLH1 (G168-728, Cell Marque, Hotsprings, USA; G168-15, BD Biosciences Pharmingen, USA), anti hMSH2 (G219-1129, Cell Marque, Hotsprings, USA; 25D12, Novocastra, UK) and anti hMSH6 (GTBP.P1/66.H6, Serotec, UK) antibodies. Biotin-streptavidine detection kit (LSAB, Dako, Glostrup, Denmark) with VIP chromogen (Vector, Burlingame, USA) and EnVision+ System (Dako Cytomation, CA, USA) with DAB substrate-chromogen were used for visualization.

1.3. DNA isolation

1.3.1. DNA isolation from paraffin embedded tissue samples of HNPCC and TGCT patients: Paraffin-embedded cancerous tissue samples of the patients were deparaffinized and DNA was extracted by the use of High Pure PCR Template Purification Kit (Roche Diagnostics, Mannheim, Germany).

1.3.2. DNA isolation from whole blood of the HNPCC patients and their relatives: DNA was extracted with the use of QIAamp DNA Blood Midi kit (Qiagen, Hilden, Germany).

1.4. Microsatellite instability analysis

Microsatellite instability test was performed on tumor samples and corresponding normal or blood samples from HNPCC suspected patients and TGCT patients. Two mononucleotide repeat markers (BAT25 and BAT26) and three dinucleotide repeat markers (D2S123, D5S346, D17S250) were studied according to the international reference panel recommendations, fluorescence labeled PCR fragments were separated and analyzed by ABI-310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA)

1.5. Mutation detection in HNPCC patients

1.5.1. PCR, heteroduplex (HD) and single strand conformation polymorphism (SSCP) analyses: DNA samples of patients with high level MSI were used to amplify all exons of the hMLH1 and hMSH2 genes. SSCP and HD analysis of PCR products were performed by electrophoresis in MDE gel (Cambrex Bio Science Rockland, Rockland, ME, USA) and visualized by silver staining.

1.5.2. Sequencing analysis: Sequencing was performed in both directions with purified PCR products using BigDye terminator cycle sequencing kit v.3.1 and the reaction products were run in ABI-PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

1.5.3. Detection of large deletions: Genomic deletions were tested by the use of SALSA MLPA Kit P003 MLH/MSH2 (MRC-Holland, Amsterdam, Netherlands) according to the manufacturer's instructions. The amplification products were analyzed by capillary gel electrophoresis (ABI-3130).

1.6. Promoter methylation analysis of hMLH1 gene in TGCT patients

Sodium bisulfite conversion of the DNA template was performed. The bisulfite reaction converts the methylation information into sequence differences. The analysis was performed by the fluorescence-based real-time PCR assay, MethyLight. The PCR reactions were performed in 7900-HT SDS (Applied Biosystems, Foster City, CA, USA). Primer and probe sets designed for bisulfite-converted DNA were used: one set for the hMLH1 promoter and a reference set for a CpG-free region of the β -actin (ACTB). SssI methylase (New England Biolabs, Ipswich, MA, USA) treated DNA was used as positive control.

These findings demonstrated that causative mutations coupled with single nucleotide polymorphisms have worse prognostic values and suggest close follow-up of carriers from their mid-twenties.

2. Influence of mismatch repair on chemoresistance of testicular germ-cell tumors

It was shown earlier that cells defective in MMR are relatively resistant to fluoropyrimidines and platinum derivatives. The majority of testicular germ cell tumors (TGCTs) are highly sensitive to cisplatin-based chemotherapy, but a small fraction of cases are resistant to the effects of chemotherapy. Although we found a strong correlation between weak or loss of hMLH1 expression and promoter hypermethylation, MSI did not correlate with either of them. Similar to several sporadic cancer cases the genetic background for MSI is unclear. Expression of the MMR proteins, hMLH1 methylation and MSI did not show correlation with the clinico-pathological parameters and the therapeutic response. According to our findings reduced expression of MMR proteins and MSI have not proved to be predictive markers for chemotherapeutic resistance of TGCTs.

3. Analysis of p53 mutations in head and neck squamous cell carcinoma (HNSCC)

The majority of the alterations we found in the core domain encoding region were missense mutations. Most of the point mutations were C:G>T:A transitions. Five out of six C:G>T:A transitions at CpG sites were found in codons of the DNA-binding surface. We can suppose that CpG site mutations affecting the DNA-binding surface have some selection advantage against those affecting the β -structure. We have not found a significant correlation between the presence of mutation and the tumor stages, suggesting that p53 mutations occur at a relatively early stage. A significant trend observed between the tumor stage progression and the frequency of missense mutations on the DNA-binding surface supports the assumption that these mutations are responsible for a more aggressive phenotype. Patients with other than missense mutations (nonsense, splice mutations, deletions) showed significantly longer survival than patients with missense mutations. Moreover, the carriers of nonsense mutations were observed to survive for the longest periods. Our results suggest the idea of dual character of the p53 gene in which missense mutations render an oncogenic property to the protein, while other types of mutations disrupt its tumor suppressor functions. We suppose that oncogenic (gain of function) properties provide a selection advantage to missense mutants.

than patients with missense mutations. All but one of the four patients with nonsense mutation were still alive 84, 88 and 96 months after the surgery. A significant linear trend was seen between tumor stage progression and proportion of subjects with mutations affecting the DNA-binding surface ($p=0.022$).

Twenty-two normal appearing resection margin samples of patients with p53 core domain mutation were also analyzed. Six (27.27%) of these samples also carried the mutation identified in the corresponding tumor samples.

3.2. SNP analysis of codon 72 of p53 gene: There were 48 R72R (53.9%), 39 R72P (43.8%) and 2P72P (2.3%) genotypes within the patient group. There were no significant differences in genotype ($p=0.622$) and allele distributions ($p=0.683$) between the patients' group and the control group of healthy individuals. There were no significant correlation between the allele distribution and the tumor stages. The overall survival curves of the R/R and R/P genotypes were not significantly different ($p=0.1490$).

DISCUSSION

1. Evaluation of genetic alterations in HNPCC suspected patients

Germline alterations of the MMR genes result in autosomal dominantly inherited predisposition to hereditary nonpolyposis colon carcinoma (HNPCC). Although HNPCC families not complying with the Amsterdam criteria show extremely low frequency of MMR gene mutations, several HNPCC patients can be missed by the obligate application of Amsterdam I and II criteria. Bethesda guidelines are applicable to select patients not fulfilling Amsterdam criteria in order to test MSI. Immunohistochemistry alone is not sufficient to use for prescreening because of its lower sensitivity, but it can be used to confirm MMR inactivation and to predict the gene being inactivated.

Among the mutation carriers two patients had very early tumor manifestation. Two germline mutations were found in each of them. A nonsense mutation and a splice mutation proved to be pathogenic respectively. The missense mutations accompanying the former alterations did not cause pathogenicity when occurred alone in family members. The presence of these polymorphisms together with the pathogenic mutations causes an early onset of tumor at the age of the twenties and early thirties of the patients, whereas relatives harboring the single pathogenic mutation developed cancer in their forties and fifties, or have not had disease yet.

1.7. Statistical analysis

Dichotomized variables gained from TGCT samples were tested by two-sided Fisher's exact test. Survival analysis was performed by using Kaplan-Meier log-rank test. Differences were considered significant at $p \leq 0.05$ significance level. The statistical tests were performed by SPSS 11.0 for Windows software (SPSS, IL, USA).

2. Analysis of p53 mutations in HNSCC patients

2.1. Patients

89 primary HNSCC and corresponding normal samples of the oral cavity (34), the oropharynx (15), the hypopharynx (23) and the larynx (17) were obtained from patients operated at the Head and Neck Surgery Department of the National Institute of Oncology, Budapest, between 1997 and 1999. Prior to surgery patients did not receive chemo- or radiotherapy. UICC stages and grades of tumors were defined. All tissue samples were snap-frozen in liquid nitrogen and stored at -75°C .

2.2. DNA isolation

DNA was isolated from the patients' samples with the standard phenol-chloroform extraction and ethanol precipitation following proteinase K digestion.

2.3. p53 mutation detection

PCRs covering the exons 5-6, exon 7 and exons 8-9 of p53 gene were performed. SSCP analysis and sequencing were performed according to the methods in *1.5.1.* and *1.5.2.*

2.4. Single Nucleotide Polymorphism (SNP) analysis

The codon 72 polymorphism of p53 gene was examined in paired tumor and normal samples of 89 HNSCC patients. PCR amplifications and melting curve analyses were performed by LightCycler instrument and software (v.3.5) (Roche Diagnostics, Mannheim, Germany).

2.9. Statistical analyses

Categorical data of two and more than two groups were compared by Fisher's exact test and χ^2 test respectively. Survival data of different populations were analyzed by Kaplan-Meier log-rank test. The above statistical tests were performed by GraphPad InStat 3 and GraphPad Prism 4 softwares. The results were considered statistically significant at $p \leq 0.05$ significance level.

RESULTS

1. Screening of hereditary nonpolyposis colorectal cancer (HNPCC) patients

1.1. Microsatellite analysis: Among the 36 patients, selected on the basis of the clinical symptoms and family history, 7 (19.4%) showed high microsatellite instability in their tumors.

1.2. Mutation detection and sequencing: We have identified germline mutations in 5 of the 7 MSI-H patients. Altogether we have found three missense, two nonsense mutations, one splice mutation and a large deletion. Two germline point mutations were found in *patient 1* and *patient 2*. In *patient 1* p.E422X nonsense mutation was accompanied by p.N127S missense mutation in hMSH2. c.2210+1G>C splice site mutation in hMSH2 and V716M missense mutation of hMLH1 were found in *patient 2*.

1.3. Immunohistochemistry: Patients having the above-mentioned mutations showed the following alterations. Cancerous tissue of *patient 1* did not express hMSH2. Tumor samples of *patient 2* showed loss of both hMSH2 and hMLH1 expressions.

1.4. Pedigree analysis: In *family 1* (family of *patient 1*) 7 persons carry the nonsense (E422X) mutations on the mother's side. Each cancerous family member bears this mutation. The missense mutation (N127S) is present in both lineages, altogether in seven persons. All family members carrying only this missense mutation are healthy. Those patients who carry the nonsense mutation only were 43 and 56 years old when colon tumors were diagnosed. In cancer patients who have both mutations tumors were manifested at the age of 32 and 34. In *family 2* (family of *patient 2*) 5 members have the splice mutation in hMSH2 (c.2210+1G>C). The missense mutation in hMLH1 (V716M) occurs in 9 persons; two family members are homozygous for this mutation. The index patient and his brother carry both alterations. Synchronous tumors were diagnosed in the index patient at the age of 25, and adenoma was found in his brother at the age of 28. Their father carrying the splice mutation only was diagnosed with cancer at the age of 52.

2. Investigation of the mismatch repair system in testicular germ-cell tumors

2.1. Immunohistochemistry: Loss or weak staining of any MMR proteins was detected in 14 cases (27.5%). Four of them belonged to the chemoresistant group. Pathological hMLH1 expression was seen in 10 cases (19.6%). In one case all of the examined MMR proteins were lost. In 4 cases

hMSSH6 protein expression was lost. Three cases with loss of hMSH6 also showed loss of hMSH2 expression. No association was found with the therapeutic response.

2.2. Microsatellite analysis: MSI was found at one microsatellite locus in 16 cases (31.4%), however no sample showed high MSI. The proportion of MSI in the refractory group and in the sensitive group was 27.8% and 32.4% respectively. The MSI status did not correlate with any of the clinicopathological parameters, the therapeutic response and not either with MMR expression.

2.3. Methylation analysis: We found hMLH1 hypermethylation in 11 cases (21.6%), of which 3 expressed hMLH1 protein strongly. However, 2 cases with loss of hMLH1 protein expression showed no hypermethylation. hMLH1 methylation was highly correlated with loss of nuclear hMLH1 expression ($p < 0.0001$) and with immunohistochemically-detected MMR deficiency ($p = 0.0005$). In addition, hMLH1 methylation was not detected in any but 1 case in the refractory group. Four deaths occurred in this series, all of them belonging to the hMLH1 nonmethylated group. However the survival curves of the hMLH1 methylated vs. nonmethylated groups did not differ significantly ($p = 0.24$). The hMLH1 methylation status did not show significant correlation with tumor stage, histology (seminoma vs. non-seminoma) and microsatellite instability.

3. Analysis of p53 mutations in head and neck squamous cell carcinoma (HNSCC)

3.1. Mutation analysis: We found 37 mutations in exons 5-8 of p53 gene, which affected 34 (38.2%) of the 89 patients examined. There were three patients who all had two mutations in this region. The most frequent changes at DNA level were C:G>T:A and A:T>G:C transitions (57.89%). The C:G>T:A transition was more than twice as frequent event as the A:T>G:C transition (39.47% vs. 18.42%). There were 25 missense (67.6%), 4 nonsense (10.8%), 1 synonym (0.02%), 2 splice site (5.4%) mutations, and 4 deletions (10.8%). Neither the overall nor the 5 year survival of patients with core domain mutations differed significantly from those without these mutations ($p = 0.63$, $p = 0.44$). Missense mutations were significantly more frequent on the DNA-contacting surface while other types of mutations occurred more often in the β -sandwich scaffold ($p = 0.03$). Patients with mutations only on the DNA-binding surface showed worse survival than those with mutations only in the β -sandwich scaffold ($p = 0.04$). Patients with other than missense mutations (nonsense, splice mutations, deletions) showed significantly longer overall ($p = 0.012$) and 5 year survival ($p = 0.020$)