Significance of genetic instability in development and chemosensitivity of malignant tumors

Ph.D. thesis

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ABBREVIATIONS

ACTB	actin beta
AIP1	ABL-interacting protein 1
APAF1	apoptotic protease activating factor 1
BAK1	BCL2 antagonist killer 1
BAX	BCL2-associated X protein
BCL2	B-cell lymphoma 2
BCL-X_l	BCL2-related protein, long isoform
BPDE	benzo[a]-pyrene diol epoxide
BRCA1	breasr cancer 1 gene
EGFR	epidermal growth factor receptor
EGR1	early growth response 1
FANCF	Fanconi anemia complementation group F
FAS	apoptosis antigen 1
FRET	fluorescence resonance energy transfer
GADD45	growth arrest and DNA damage inducible gene 45
HD	heteroduplex
HDM2	human double minute 2 homolog
hMLH1, hMLH3	human Mut L homologue 1 and 3
hMSH2, hMSH6	human Mut S homolgogue 2 and 6
HNPCC	hereditary nonpolyposis colon carcinoma
HNSCC	head and neck squamous cell carcinoma
hPMS1, hPMS2	human postmeiotic segregation increased S. cerevisiae homolog 1 and 2
HPV	human papillomavirus
IARC	International Agency for Research on Cancer
LOH	loss of heterozygosity
MDR1	multidrug resistance 1
MLPA	multiple ligation dependent probe amplification
MMR	mismatch repair
MSI	microsatellite instability
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PMR	percent of methylated reference
PUMA	p53 upregulated modulator of apoptosis
SNP	single nucleotide polymorphism
SSCP	single strand conformation polymorphism
TBS	tris buffered saline
TGCT	testicular germ-cell tumor
UICC	International Unioin Against Cancer
5-FU	5-fluorouracil

INTRODUCTION

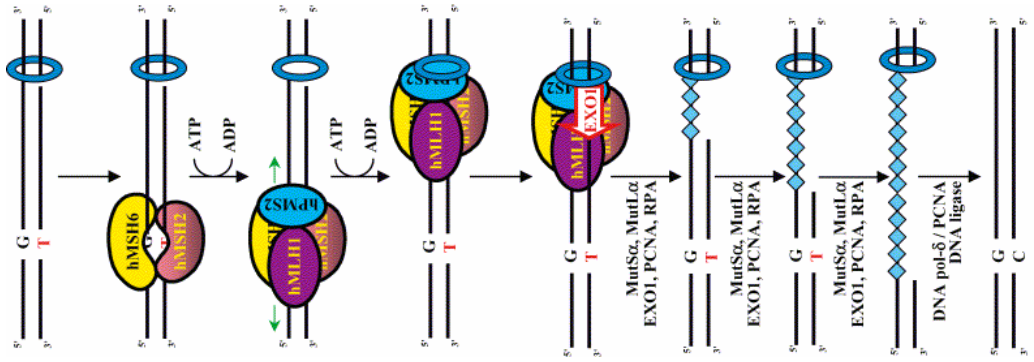
1. Genetic instability and cancer

Cancer is a disease resulting from the accumulation of genetic alterations in the cell. Exposure to environmental carcinogens (chemicals, viruses, radiations) contributes to the genesis of at least 80% of all human cancers (1). There are three possible outcomes for a cell following exposure to DNA damage: (a) the cell could repair the damage; (b) the cell could die; or (c) survive with a permanent mutation resulted by replication of the damaged DNA. DNA damage elicits cell cycle arrest at G1/S or G2/M transitions allowing to repair the errors occurred during DNA replication or chromosome segregation (2). The p53 protein acts as a checkpoint control to permit the repair of damaged DNA by cell cycle arrest in G1. (3) Genes involved in the maintenance of genomic integrity - such as DNA- mismatch repair genes, p53 and BRCA1 - are called also as „care taker" genes. Defects in the cellular mechanisms protecting against DNA damages increase the genetic instability therefore lead to the accumulation of genetic errors, which may eventually result in tumor development. The inactivation mechanisms may be inherited alterations, acquired mutations and epigenetic events.

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. At present seven MMR genes are known (MLH1, MLH3, MSH2, MSH3, MSH6, PMS1, PMS2). In human cells, mismatch recognition is performed by hMSH2 heterodimerized either with hMSH6 (MutSα complex) for base-base mismatches and loops of one or a few nucleotides or with hMSH3 (MutSβ complex) for insertion/deletion of two or more extrahelical bases. These complexes then interact with another heterodimeric complex, composed of hMLH1 and hPMS2 (4). Exonuclease I mediates excision of the newly synthesized daughter strand extending from a nick to the mismatch (5). DNA-polymerase δ / PCNA complex then resynthesizes the degraded lesion leaving a nick that can be sealed by a DNA-ligase (6). Figure 1 schematically represents the MMR in human cells.

Figure 1 (ref. 7)



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) (8, 9). This syndrome is characterized by early onset colorectal carcinoma and extracolonic epithelial-derived tumors most often located in the gastrointestinal and the urogenital tracts (10). Affected individuals inherit a mutation in one of the alleles and when a second mutation is acquired in the wild-type allele, the cell is less able to repair DNA mismatch errors (10). In the majority of HNPCC cases, germline mutations of the hMLH1 and/or hMSH2 genes can be demonstrated (11). 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. This phenomenon can be utilized as a diagnostic marker in screening of HNPCC suspected patients (12). Amsterdam criteria (I and II) and Bethesda guidelines serve as bases for patient selection (13, 14, 15). If the diagnosis of HNPCC is supported by immunohistochemistry and microsatellite instability analysis identification of genetic alterations makes possible further screening of other family members.

An alternative way of gene inactivation is promoter hypermethylation. Methylation of cytosines located 5' to guanosine in CpG-rich regions, so called CpG islands of gene promoters, especially accompanied by histone deacetylation and other modifications, makes chromatin structure inaccessible to cellular transcription machinery (16). In the case of MMR system, 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 (17).

MMR deficiency confers cancer predisposition not only through failed repair of DNA mismatches but also through the failure to signal apoptosis in cells with damaged DNA. The

molecular effects of many carcinogenic insults resemble those caused by cytotoxic agents, suggesting that the cellular response to carcinogenic agents and chemotherapeutics are similar (18). Cells defective in either hMutS homologs or hMutL homologs fail to engage chemical induced apoptosis. (18). Therapeutic drugs modify DNA to form adducts, which could introduce mispairs during DNA replication. hMut homologs can recognize these mismatches to provoke a strand-specific MMR reaction. Since MMR is always targeted to the newly synthesized strand, the offending adducts, which are located in the template strand, cannot be removed by strand–specific MMR and continue to produce unusual base pairs upon DNA resynthesis. Such futile repair may be translated into an apoptotic signal through both p53-dependent and p53-independent pathways. Cisplatin induces intrastrand crosslinks which are recognized but not processed by the MMR system. (19). DNA-cisplatin adducts lead to replication arrest, cell cycle checkpoint activation and sustained G2 arrest and if the damage is too severe, cell death. Cell lines that have lost expression of hMLH1 or hMSH2 are 2- to 4-fold more resistant to cisplatin. (20). 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 mismatch repair enzymes is associated with resistance to antracyclines and fluoropirimidines (21). On the other hand, some papers reported that MMR defective colorectal carcinoma cells exhibited increased sensitivity to topoisomerase I poisons: camptothecin and irinotecan (22, 23).

1.1.2. Chemosensitivity of testicular germ cell tumors and the mismatch repair

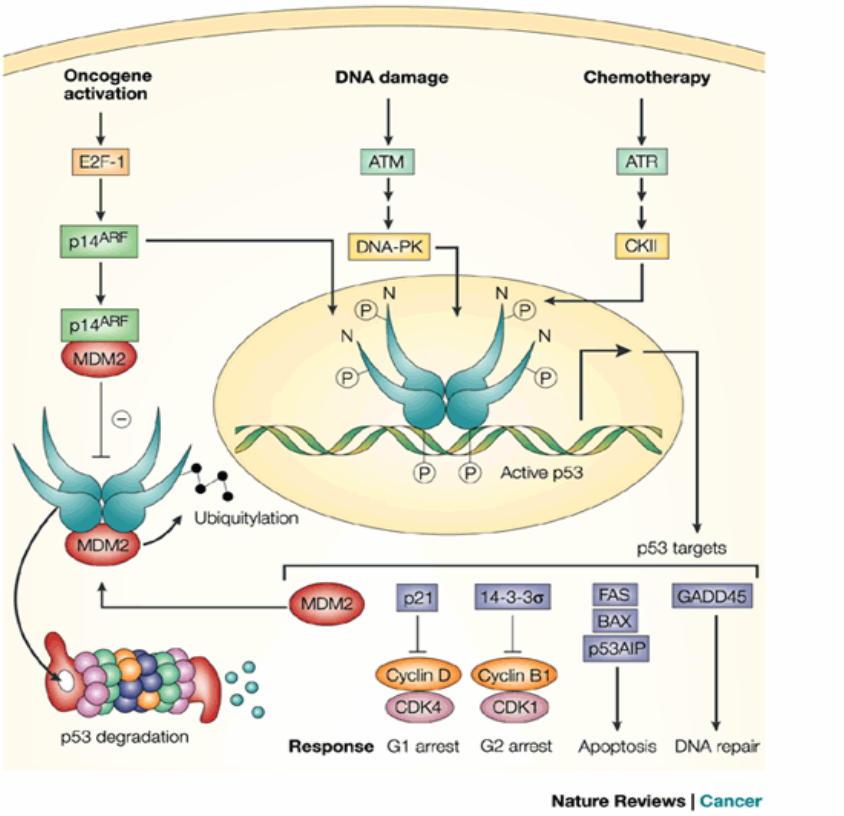
Testicular germ-cell tumors (TGCT) are highly sensitive to chemotherapy. Even in patients with metastatic disease, cure rates of 80% can be achieved with cisplatin based chemotherapy (24). Based on histological and clinical characteristics germ cell tumors (GCT) can be divided into seminomas and nonseminomas. Seminomas are uniform cells resembling primordial germ cells. Nonseminomas contain one or more histological subtypes representing various differentiation lineages and stages of development. Embryonal carcinoma cells represent the stem cell component, which has the potential to differentiate towards extra-embrionic tissues (yolk sac tumor and choriocarcinoma) and embrionic tissues with mesenchymal, epithelial or neuronal components (immature and mature teratoma) (25). Mature teratomas are clinically resistant to the chemotherapy. Due to intrinsic chemoresistance, mature teratoma can be found in 30-40% of residual lesions after chemotherapy of non-seminomatous GCTs (26). 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 found that 45% of tumor samples from patients with refractory disease had unstable microsatellites, suggesting that failure to initiate apoptosis due to defects in MMR might contribute to resistance (27).

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. It also responds to several cellular stresses, including oncogene activation and hypoxia. In normal cells p53 is inactivated by HDM2, an ubiquitin ligase that targets p53 for proteosomic degradation and conceals its transactivation domain. Several types of stress can activate p53 by posttranslational modifications leading to dissociation of the p53-HDM2 complex. Activated p53 can induce target genes involved in cell cycle arrest (e.g. $p21^{WAF1}$), apoptosis (e.g. Fas, Bax, Bak, AIP1, PUMA), or DNA repair (e.g. GADD45) (28), and it can repress antiapoptotic genes (e.g. Bcl-2, Bcl-X_L, survivin) (29, 30). The scheme of the main pathways of p53 functions can be seen in Figure 2.

Figure 2 (ref. 31)



Inactivating mutations in p53 are the most common genetic alterations found in human cancer. More than half of all human cancers lose the p53 function by mutation (31). 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 (32). Therefore mutant p53 presents an important target for drug intervention.

1.2.1. Mutations disrupting the p53 function

The p53 protein as being a transcription factor bears an amino-terminal transactivation domain, a core DNA-binding domain, and carboxy-terminal tetramerization and regulatory domains (31). The majority of tumor-derived mutations map to the central DNA binding domain, nevertheless 13.6% of mutations are located outside this region (33). The mutations in the DNA-binding domain lead to the inability of p53 to bind DNA and transactivate p53-target genes. More than 75% of these mutations occur as single missense mutations rather than deletions, insertions or frameshifts (31). These missense mutations lead to the synthesis of a stable, but inactive protein that accumulates in the nucleus of tumor cells (34). On the contrary, frameshift and nonsense mutations do not result in accumulation and can not be detected by immunohistochemistry. The structure of the core domain consists of a β-sandwich structure and a DNA-binding surface. Several studies found that mutations on the DNA-binding surface cause poorer prognosis or treatment response than mutations in the β-sandwich scaffold (36, 37, 38).

1.2.2. Dominant negative and oncogenic effects of mutant p53

Missense mutations in p53 may result not only in loss of function, but also in dominant negative and oncogenic („gain of function") effects. P53 protein exerts its effect as a homotetramer. The dominant negative effect corresponds to the capacity of the mutant protein to complex with the product of the remaining wild-type allele to inactivate its function (39). Two additional p53 family members, p63 and p73 have recently been characterized (40). Both contain regions that correspond to the amino-terminal transactivation, central DNA-binding and carboxy-terminal oligomerization domains of p53. P63 and p73 proteins can bind to the consensus sequences of p53, activate transcription of several p53 target genes, and induce apoptosis when overexpressed. Although wild-type p53 cannot bind to p63 and p73, some p53 mutants can form hetero-tetramers with them leading to inactivation their ability to induce

apoptosis. Some papers have demonstrated that the drug resistance associated with overexpression of gain of function p53 mutants is at least in part due to their inhibition of p73 activity (41, 42). The binding affinity of mutant p53 to p73 is influenced by the status of a common p53 polymorphism at residue 72. The interaction is enhanced when p53 codon 72 encodes an arginine (R) rather than a prolin (P) (43). This stronger binding by mutant p53 R72 proteins is associated with an increased ability to inhibit p73-dependent apoptosis. In a recent study it has been reported that p53 R72 mutants are more potent inhibitors of chemotherapy-induced apoptosis (41). Bergamaschi and co-workers demonstrated that patients with head and neck squamous cell carcinoma (HNSCC) those whose tumors expressed the R72 allele of mutant p53 had both a decreased response to cisplatin treatment and an overall worse survival than those expressing the 72P allele (41). These results create a link between codon 72 polymorphism, p73 binding, chemosensitivity and survival.

The oncogenic effects of mutant p53 may partly be related to that p53 mutants seem to transactivate or repress specific genes. In reporter assays core domain mutants were found to transactivate promoters of specific target genes, such as MDR1, c-myc, PCNA and EGFR (Reviewed in ref. 44).

1.2.3. p53 – a prognostic marker in head and neck squamous cell carcinoma

P53 mutation is one of the most common genetic changes in head and neck squamous cell carcinoma (HNSCC) (45). Some studies have reported an association between smoking and alcohol use and the frequency of p53 mutations (46). Early studies suggested that p53 alterations are early events in some HNSCC, others identified an increase in the incidence of p53 mutation with progression of HNSCC from a non-invasive to an invasive phenotype (47). In a subset of HNSCC lacking p53 mutations, p53 function is compromised by the interaction with the E6 protein encoded by oncogenic HPV types, especially HPV 16 and HPV 18 (48).

Molecular genetic and immunohistochemical alterations of p53 could be useful diagnostic markers in the management of HNSCC. P53 mutations could be detected by an early study in pre-operative saliva samples from HNSCC patients. (49). Analysis of p53 mutations may also allow the differentiation between HNSCC originated from a single neoplastic lesion and those arised from an independent second primary tumor (50). Loco-regional recurrence, despite apparently adequate resection, occurs in up to 30% of surgically managed HNSCC (45), presumably reflecting the presence of residual malignant cells in the surgical margins which are not detected by histopathology. Therefore detection of p53 mutations in apparently cancer-free surgical margins is valuable in identification of patients in whom loco-regional recurrence

is probable (51). Detection of p53 mutations may also be useful in prediction of response of patients treated with chemotherapy. Temam et al. showed that the presence of p53 contact mutations conferred a high risk treatment failure in patients treated with cisplatin and 5-fluorouracil-based induction therapy (52). Other studies demonstrated that mutations in p53 are strongly associated with loco-regional treatment failure following radiotherapy (53).

Abundant data support an important role for p53 in tumor genesis of the squmaous epithelium of the head and neck. Analysis of alterations in the p53 gene obviously has diagnostic and prognostic utility in HNSCC. It is hoped that this will facilitate prediction of treatment response of individual HNSCC patients. Now new therapeutic strategies are emerging which specifically target p53 deficiency. Some of them are aimed at restoration of wild type function using small molecules that induce mutant p53 to adopt a wild-type conformation (54). Another approach is the application of siRNA to downregulate the expression of the mutant p53 without affecting the wild-type p53 (55). SiRNA can be tailored to a patient's specific p53 mutation, and in combination with traditional chemotherapies would increase the likelihood of tumor response to treatment.

THE AIMS OF THE STUDY

Exposure to environmental carcinogens could result in genetic and epigenetic alterations. Without adequate cellular responses, such as cell-cycle arrest, DNA repair, or apoptosis, genetic errors accumulate and a pattern of methylated promoters evolves as a result of clonal selection. A synergy between genetic and epigenetic alterations drives the tumor progression. Inherited abnormalities in genes involved in DNA damage response (HNPCC, Li-Fraumeni syndrome) significantly increase the cancer susceptibility of mutation carriers.

Our aims in this study were to examine the main mismatch repair (MMR) genes and p53 gene as representatives of "care takers". Inactivations of these genes are early events in carcinogenesis, have prognostic value in cancer development, and also serve as predictive markers for therapeutic sensitivity.

The following issues were set for investigation:

A) Examination of 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. Examination of mismatch repair deficiency in TGCTs as a predictive marker of chemotherapeutic sensitivity.
   2.1. Assessment of hMLH1, hMSH2 and hMSH6 protein expressions in testis tumors.
   2.2. Analysis of the correlation between hMLH1 protein expression and promoter hypermethylation.
   2.3. Examination of the relation between the microsatellite instability (MSI) and the expression of MMR proteins.
   2.4. Correlation analysis of cisplatin resistance with the expression of MMR proteins and also with the MSI status.

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).

1.1. Identification of mutations occurring in the exons encoding the core domain of the p53 protein. Correlation analysis between the p53 mutations and the clinico-pathological parameters. Assessing the effects of different types of mutations on the survival of the patients.

1.2. Assessment of the renewal risk of HNSCC tumors depending on the presence or absence of the p53 mutations in the resection margins.

2. Comparison of the germline allele and genotype distribution of R72P polymorphism of p53 between the HNSCC patients and a healthy control population. Correlation analysis of the codon polymorphism with the patient survival and the p53 mutations.

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. *Patient 1* was 32 years old and *patient 2* was 25 years old at the time of diagnosis. 11 family members of patient 1 and 14 family members of patient 2 were examined in further mutational analysis. The index patients met the criteria summarized in Annex 1.1.

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 (21). Staging was based on UICC classification (58). Early stage was defined as stage I or stage II/A. Late stage was defined as stage II/B, II/C or stage III. Therapy was performed according to the institution's protocol (59). The clinical response was measured in accordance with accepted criteria in testis tumors (60). 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. Patients' data are summarized in Annex 1.2.

1.2. Immunohistochemistry

1.2.1. Immunohistochemistry of HNPCC samples: 5 μm thick paraffin embedded tissue sections were deparaffinized with xylene, rehydrated in decreasing concentrations of ethanol, washed in distilled water and microwaved for 20 minutes in citrate buffer (pH 6.4). Non-specific binding was blocked by 3% bovine serum albumin in PBS. Sections were then incubated with the following primary monoclonal antibodies for 1 hour in room temperature: mouse anti-hMLH1 (G168-728, Cell Marque, Hotsprings, AR, USA) and mouse anti-hMSH2 (G219-1129, Cell Marque, Hotsprings, AR, USA). The negative control was processed in the same way but with the omission of the primary antibodies. The sections were washed with PBS and incubated with secondary antibody using biotin-streptavidine detection kit (LSAB, Dako, Glostrup, Denmark) with VIP chromogen (Vector, Burlingame, CA, USA). Negativity

was declared in the absence of any nuclear signal in tumor cells. Nuclei were counterstained with methyl green (Dako, Glostrup, Denmark).

1.2.2. Immunohistochemistry of TGCT samples: 4 μM thick formalin-fixed paraffin-embedded tumor sections were deparaffinized with xylene, rehydrated in decreasing concentrations of ethanol, washed in distilled water. Endogenous peroxidase blocking was performed with 3% H_2O_2. The slides were washed with distilled water and rinsed in citrate puffer (pH 6.0). Heat induced epitope retrieval was performed in water bath (97 ^oC, 35 min). The slides were incubated in TBS (1h, room temperature) with mouse primary monoclonal antibodies: anti-hMLH1 (G168-15, BD Biosciences Pharmingen, CA, USA); anti-hMSH2 (25D12, Novocastra, UK) and anti-hMSH6 (GTBP.P1/66.H6, Serotec, UK). The sections were washed in TBS, and then incubated with polymer-horseradish peroxidase (EnVision + System, DakoCytomation, CA, USA) for 30 minutes. The slides were washed in TBS, were incubated with DAB substrate-chromogen (DakoCytomation, CA, USA) and slightly counterstained with Mayer's hematoxylin. Paraffin-embedded human tonsil tissue was used as positive control. The primary antibody was replaced with 3% bovine serum albumin in the case of the negative control.

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 first deparaffinized with xylol, then rehydrated with ethanol. 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), according to the manufacturer's protocol.

1.4. Microsatellite instability analysis

Microsatellite instability test was performed on tumor samples and corresponding blood or normal tissue 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 (56). Primer sequences were chosen from the Human Genome Database (http://www.gdb.org) (Annex 2), sense primers were labeled fluorescently. The PCR fragments were separated by ABI-310 Genetic Analyzer (Applied Biosystems, Foster City,

CA, USA) and the analysis was performed with GeneScan 3.7 software (Applied Biosystems, Foster City, CA, USA). The MSI of colon cancer samples was assessed according to the consensus of the National Cancer Institute workshop on microsatellite instability for colorectal cancer detection (56). High level instability (MSI-H) was diagnosed when more than 30% of the examined markers showed new alleles in the tumor tissue compared to the blood control. MSI positivity was defined in TGCT samples if a new marker peak appeared comparing with the normal sample.

1.5. Mutation detection in HNPCC patients

1.5.1. PCR, heteroduplex (HD) and single strand conformation polymorphism (SSCP) analyses: DNA samples isolated from blood of patients with high level MSI were used to amplify all exons of the hMLH1 and hMSH2 genes. Primer sequences are listed in Annex 3.1. Following denaturation (SSCP) or heteroduplex formation (HD) PCR products were subjected to electrophoresis in MDE gel (Cambrex Bio Science Rockland, Rockland, ME, USA) according to the manufacturer's instructions and visualized by silver staining (57).

1.5.2. Sequencing analysis: Sequencing was performed in both directions with purified PCR products showing altered migration pattern in the MDE gel. The sequencing reactions were carried out using BigDye terminator cycle sequencing kit v.3.1 (Applied Biosystems, Foster City, CA, USA) 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). A deletion of one copy of a probe target sequence was stated if the relative peak area for that probe amplification product had been reduced by 35-55% compared to a negative control sample.

1.6. Promoter methylation analysis of hMLH1 gene in TGCT patients

Sodium bisulfite conversion of the DNA template was performed as described previously (61). This method is based on a chemical modification of cytosine residues in the presence of sodium bisulfite. In the first step of the bisulfite reaction, cytosines are sulfonated and deaminated converting them to uracil sulphonate. A subsequent desulfonation at a basic pH completes the conversion from cytosines to uracils. C5-methyl-cytosines are not modified under the conditions used, so the bisulfite reaction effectively converts methylation

information into sequence differences. The methylation analysis was performed by the fluorescence-based real-time PCR assay, MethyLight, as described previously (62) The PCR reactions were performed in ABI 7900-HT instrument (Applied Biosystems, Foster City, CA, USA). Primer and probe sets, designed specifically for bisulfite-converted DNA, were used: one set of primer pair and TaqMan probe specific for a fully methylated CpG island of the hMLH1 promoter and a reference set for a CpG-free region of the β-actin (ACTB) gene to normalize for input DNA (Annex 4). Human sperm DNA was used as negative control and human lymphocyte DNA treated with SssI methylase (New England Biolabs, Ipswich, MA, USA) was used as positive control. The percentage of fully methylated molecules, that is percent of methylated reference (PMR), was calculated by dividing of the hMLH1:ACTB ratio of the sample by the hMLH1:ACTB ratio of the positive control and multiplying by 100 (62). A sample was assessed hypermethylated if the result reached a minimum of 5 PMR, as a threshold of 5 PMR gave the most significant discrimination between the tumor and the normal samples. The primers and probes used in the assay were described elsewhere (63) (Annex 4). The PCR reactions were performed in 1x JumpStart Taq ReadyMix (Sigma-Aldrich, St. Louis, MO, USA).

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. Examination 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 (58) and grades of tumors were defined. Patient data are detailed in Annex 1.3. There were 58 histopathologically normal appearing resection margin samples available. All tissue samples were snap-frozen in liquid nitrogen and stored at $-80\ ^{0}C$.

2.2. DNA isolation

DNA was isolated from the patients' samples with the standard phenol-chloroform extraction and ethanol precipitation following proteinase K (Sigma-Aldrich, St. Louis, MO, USA) digestion.

2.3. p53 mutation detection

PCRs covering the exons 5-6, exon 7 and exons 8-9 of p53 gene were performed by primers described in Annex 3.2. 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. The genotypes were determined by melting curve analysis using LightCycler instrument and software (v.3.5) (Roche Diagnostics, Mannheim, Germany). Amplification and detection were performed using LightCycler FastStart DNA Master HybProbe mix (Roche Diagnostics, Mannheim, Germany) with primers and fluorescent hybridization probes detailed in Annex 5. The sequences of the two probes enable them to hybridize to the amplified target sequence in a head-to-tail arrangement, bringing the two dyes into close proximity when fluorescence resonance energy transfer (FRET) and fluorescence emission can take place. During the melting curve analysis the different genotypes can be distinguished as the probe perfectly matching target DNA requires a higher melting temperature (T_m) to separate than that one bound to DNA containing a single base mismatch (corresponding to the SNP). Therefore the fluorescence intensity drops at different temperatures during a slow temperature increase of the PCR products of different allelotypes.

2.5. Statistical analysis

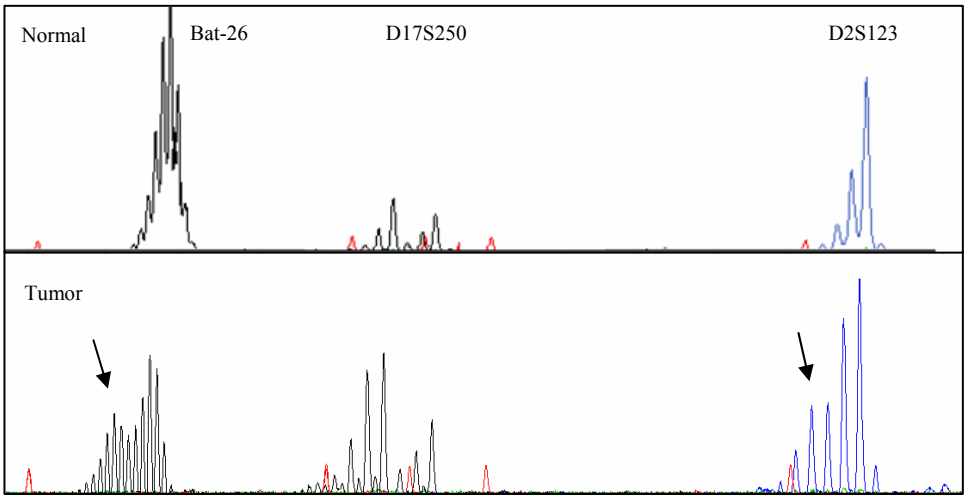
Associations of the p53 mutation and codon 72 polymorphism with the pathological parameters (tumor, node, stage, grade) and also with the tumor sites were assessed using χ^2 tests. The germline genotype distributions of the patients and a healthy population of 216 individuals were compared by the use of χ^2 test. Association between codon 72 polymorphism and pathogenic mutations were analyzed with Fisher's exact test. Connections of different mutations or codon 72 polymorphism with survival were analyzed by Kaplan-Meyer log-rank tests. The above statistical tests were performed by GraphPad Instat 3 and GraphPad Prism 4 softwares.

RESULTS

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

1.1. Microsatellite analysis: Among the 36 selected patients 7 (19.4%) showed high microsatellite instability in their tumors, that is two or more of the five tested microsatellite markers were unstable. Figure 1 demonstrates a representative microsatellite unstable sample (*patient 1*).

Figure 1



1.2. Mutation detection and sequencing: We have identified germline mutations in 5 of the 7 MSI-H patients. The characteristics of the mutations are summarized in Table 1.

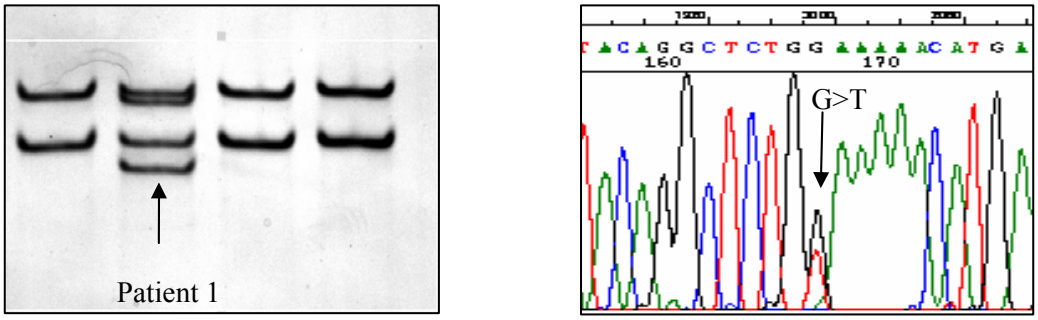
Table 1

Patient (age)	***Gene***	***Mutation***	***Domain***	***Consequence***	
1 *(32 y)*	hMSH2	c.1264G>T	hMSH3/hMSH6 interaction	p.E422X	nonsense
	hMSH2	c.380A>G	DNA binding	p.N127S	missense
2 *(25 y)*	hMSH2	c.2210+1G>C	MutL homologs interaction	Splicing error	frameshift
	hMLH1	c.2146G>A	hPMS2/hMLH3/hPMS1 interaction	p.V716M	missense
3 *(36 y)*	hMLH1	c.143A>C	ATP-ase	p.Q48P	missense
4 *(44 y)*	hMSH2	c.2131C>T	Walker A	p.R711X	nonsense
5 *(49 y)*	hMLH1	g.26844_28630del1787	MutS homologs interaction		frameshift

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

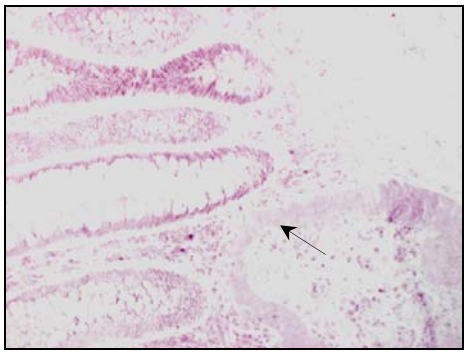
patient 1 p.E422X nonsense mutation was accompanied by p.N127S missense mutation in hMSH2. c.2210+1G>C splice site mutation in hMSH2 and V716M missense mutation of hMLH1 were found in *patient 2*. Figure 2 demonstrates the SSCP pattern and the sequencing of the nonsense (p.E422X) mutation of hMSH2 in *patient 1*.

Figure 2



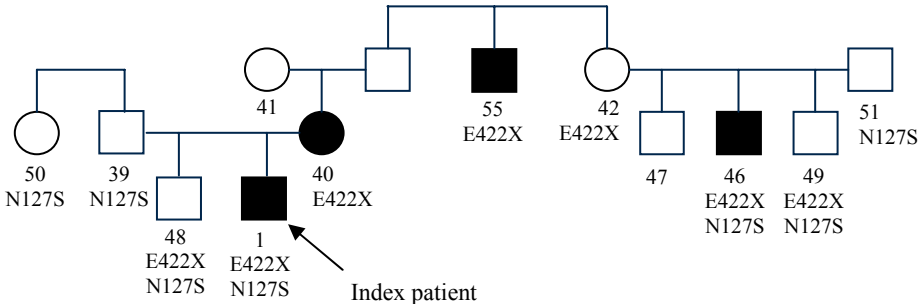
1.3. Immunohistochemistry: Cancerous tissue of *patient 1* and *patient 4* did not express hMSH2. Tumor samples of *patient 2* showed loss of both hMSH2 and hMLH1 expression. *Patient 5* have positive staining of hMLH1 and hMSH2 in his tumor sample. We have no immunohistochemical data of *patient 3.* Figure 3 represent the immunohistochemistry of a tissue section from *patient 1*. The arrow indicates the loss of staining of hMSH2 in the cancerous region of the section.

Figure 3



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. Figure 4 represents the pedigree of *patient 1*. Black symbols indicate cancer patients.

Figure 4



Four family members bare both mutations; two of them have colon cancer. 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 (*patient 1*) and 34 (*patient 46*). The other two brothers with double mutation were healthy at the age of 28 (*person 48*) and 31 (*person 49*). These data are shown in Table 2.

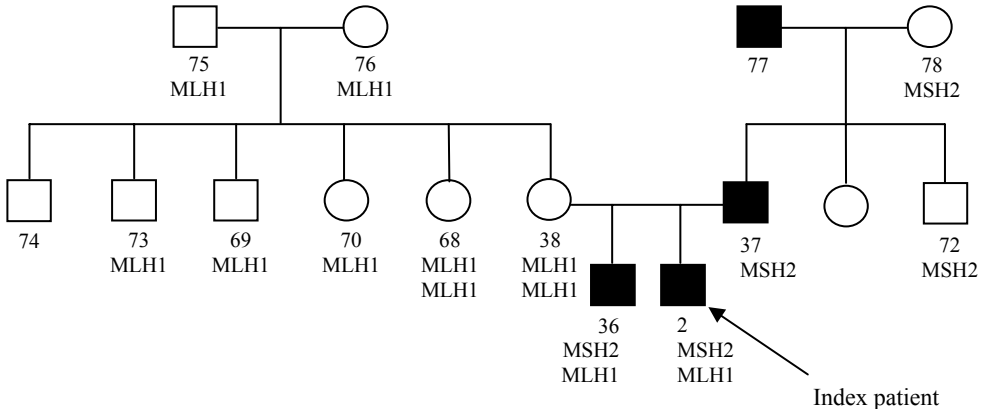
Table 2

Family members	***Nonsense (E422X) mutation in hMSH2***	***Missense (N127S) mutation in hMSH2***	***Age at diagnosis (years)***
1 (index)	+	+	32
46	+	+	34
40	+	-	43
55	+	-	56

In *family 2* (family of *patient 2*) 5 members have the splice mutation in hMSH2 (c.2210+1G>C): the index patient, his brother, his father, the brother of his father and his paternal grandmother. The missnese mutation in hMLH1 (V716M) occurs in 9 persons; two family members (persons 38 and 68) 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 was diagnosed with cancer at the age of 52. The paternal grandfather, who did not carry mutations in hMLH1 and hMSH2, developed colorectal cancer over the age of 80. The examined family members and the identified mutations can be seen in Figure 5.

The altered genes are indicated under the symbols. Black symbols indicate cancer patients.

Figure 5



The identified mutations and the ages of patients at the time of diagnosis are summarized in Table 3.

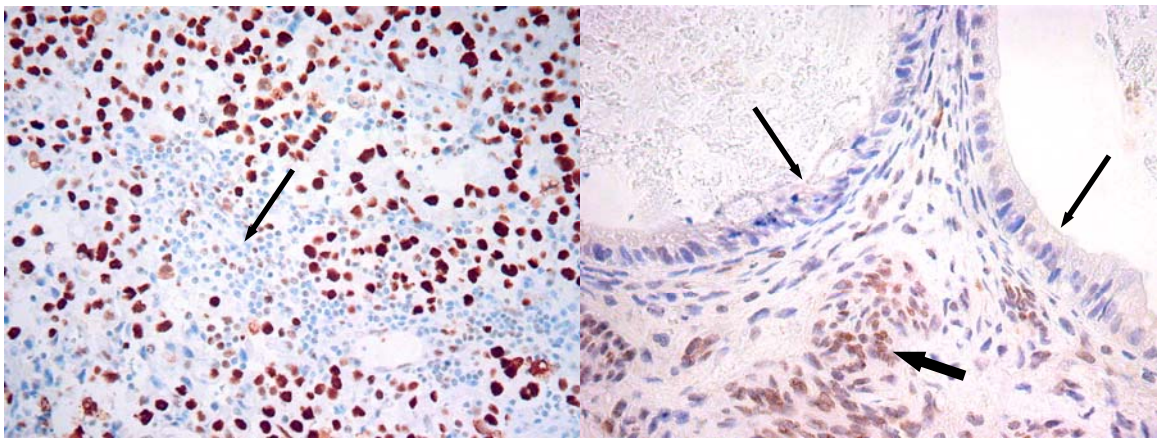
Table 3

Family members	***Splice site mutation (c.2210+1G>C) in hMSH2***	***Missense (V716M) mutation in hMLH1***	***Age at diagnosis (years)***
2 (index)	+	+	25
36	+	+	28 (adenoma)
37	+	-	52
72	+	-	?

2. <u>Investigation of the mismatch repair system in testicular germ-cell tumors</u>

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. In cases with intact intranuclear reactivity, the intensity of staining was higher than in normal stromal lymphocytes (indicated by an arrow in Figure 6A). Pathological hMLH1 expression was seen in 10 cases (19.6%) (Figure 6B). In one case all of the examined MMR proteins were lost. In 4 cases hMHSH6 protein expression was lost. Three cases with loss of hMSH6 also showed loss of hMSH2 expression. We found no isolated hMSH2 expression (Table 4). No association was found between the expression of MMR proteins and the therapeutic response.

Figure 6



A: Seminoma. Strong intranuclear staining of hMSH2 in tumor cells compared to infiltrating normal lymphocytes

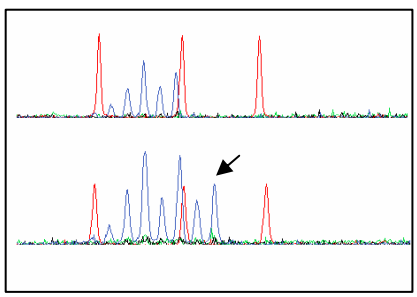
B: Mature teratoma. Loss of hMLH1 in in the epithlial component (thin arrows). Normal hMLH1 expression in the stromal component (thick arrow).

Table 4

Loss of protein expression	***Number of cases***	***Cisplatin resistant***
hMLH1	9	3
hMSH6	1	-
hMSH6 and hMSH2	3	-
hMSH6, hMSH2, hMLH1	1	1
Any of the MMR proteins examined	14 (27.5%)	4

2.2. Microsatellite analysis: MSI was found at one microsatellite locus in 16 cases (31.4%), however no sample showed high MSI. Figure 7 demonstrates MSI at D17S250 microsatellite marker.

Figure 7



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 clinico-pathological parameters investigated (tumor stage, histology, resistance to systemic treatment) 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 ($p=0.0005$). Correlation of hMLH1 protein expression and hMLH1 methylation is summarized in the following table.

Table 5

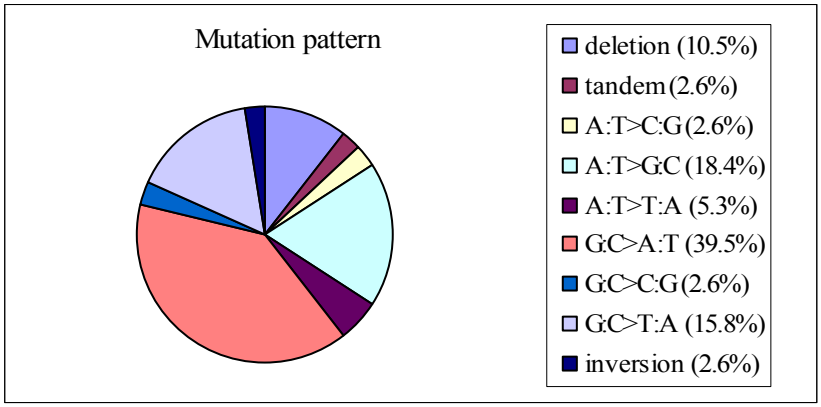
hMLH1 promter methylation	***hMLH1 protein expression***		***Total***	***p***
	Weak / absent	***Strong***		
Positive	8	3	11	<0.0001
Negative	2	38	40	
Total	10	41	51	

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)

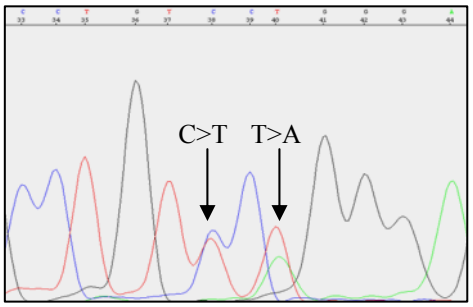
3.1. Mutation analysis: We found 37 mutations in exons 5-8 of p53 gene, which affected 34 (38.2%) of the 89 patients examined. (Patients data are detailed in Annex 1.3). There were three patients who all had two mutations in this region. The mutation pattern is demonstrated in Figure 8. Tandem mutations were detected in patient 131 at codon 161 (GCC>TTC). In patient 119 substitutions of C>T and T>A were detected in codon 278 (CCT>TCA) (Figure 9).

Figure 8



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%). Two of the deletions were out of frame deletions.

Figure 9



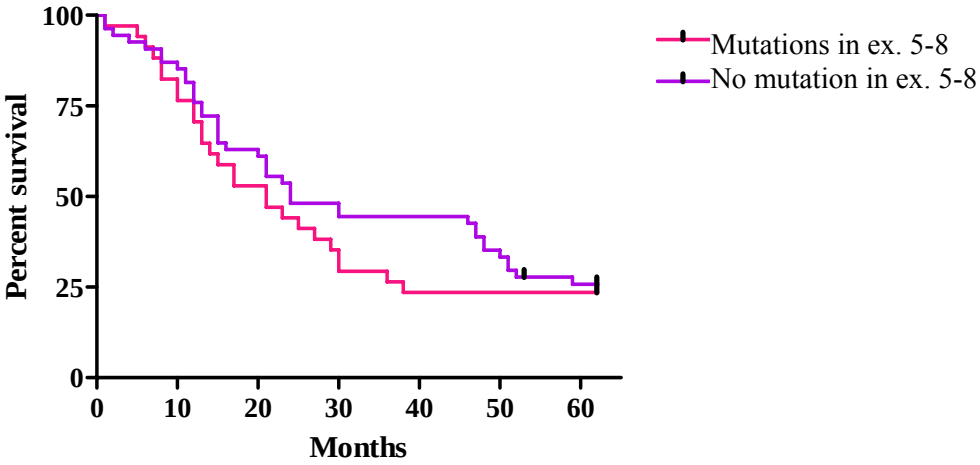
Among the 25 missense mutations 18 (72.0%) were located on the DNA binding surface and 7 (28%) in the β-sandwich structure. Three of the nonsense mutations and three of the deletions were found in the β-sandwich, and one of each on the DNA-binding surface. Two of the mutations near by splice sites were also located on the DNA-binding surface. The identified mutations are characterized in the following table.

Table 6

	Sample	*Mutation*	*Consequence*	*Core domain element*	*Element function*
Oral cavity	4	c.734G>T: G245V	missense	L3	DNA-binding (stabilizing)
	18	c.844C>T: R282W	missense	H2	DNA-binding (stabilizing)
	120	c.821T>G: V274G	missense	S10	DNA-binding (stabilizing)
	125	c.814G>A: V272M	missense	S10	DNA-binding (stabilizing)
	72	c.818G>A: R273H	missense	S10	DNA-binding (contact)
	16	c.476C>T: A159V	missense	S4	β sandwich – structural scaffold
	85	c.614A>G: Y205C	missense	S6	β sandwich – structural scaffold
	131	c.481G>T,482C>T: A161F	missense	S4	β sandwich – structural scaffold
	50	c.661G>T: E221X	nonsense	S7-S8 loop	β sandwich – structural scaffold
	110	c.637C>T: R213X	nonsense	S6-S7 hairpin	β sandwich – structural scaffold
	9	c.639A>G: R213R	synonym	S6-S7 hairpin	β sandwich – structural scaffold
	42	c.673-3_681del12	in frame deletion	S7-S8 loop	β sandwich – structural scaffold
	133	c.919+1G>A	splicing error, frameshift		nuclear localization
Oropharynx	41	c.742C>T: R248W	missense	L3	DNA-binding (contact)
	70	c.713G>A: C238Y	missense	L3	DNA-binding (Zn^{2+}-binding, stabilizing)
	114	c.747G>T: R249S	missense	L3	DNA-binding (stabilizing)
	119	c.832C>T,834T>A: P278S	missense	H2	DNA-binding (stabilizing)
	127	c.530C>T: P177L	missense	L2	DNA-binding (stabilizing)
	135	c.536A>G: H179R	missense	L2	DNA-binding (Zn^{2+}-binding, stabilizing)
	92	c.438_443del6	in frame deletion	S3	β sandwich – structural scaffold
Hypopharynx	3	c.434G>T: G245V	missense	L3	DNA-binding (stabilizing)
	106	c.818G>A: R273H c.432_538del107	missense out of frame deletion	S10 S3	DNA-binding (contact) β sandwich – structural scaffold
	84	c.395A>T: K132M c.659A>G: Y220C	missense missense	S2' S7-S8 loop	DNA-binding (contact) β sandwich – structural scaffold
	112	c.384_402del19	out of frame deletion	S2-S2' hairpin	DNA-binding (stabilizing)
	19	c.464C>A: T155N	missense	S3-S4 loop	β sandwich – structural scaffold
	104	c.560+1G>A	splicing error, frameshift	L2	β sandwich – structural scaffold
	117	c.659A>G: Y220C	missense	S7-S8 loop	β sandwich – structural scaffold
Larynx	58	c.743G>A: R248Q	missense	L3	DNA-binding (contact)
	81	c.848G>C: R283P	missense	H2	DNA-binding (contact)
	94	c.734G>A: G245D	missense	L3	DNA-binding (stabilizing)
	149	c.574C>T: Q192X	nonsense	L2	DNA-binding (stabilizing)
	71	c.578A>G: H193R c.561-3-(-2)TA>AT	missense splicing error?	L2 L2	DNA-binding (stabilizing)
	12	c.702C>A: Y234X	nonsense	S8	β sandwich – structural scaffold
	87	c.659A>G: Y220C	missense	S7-S8 loop	β sandwich – structural scaffold

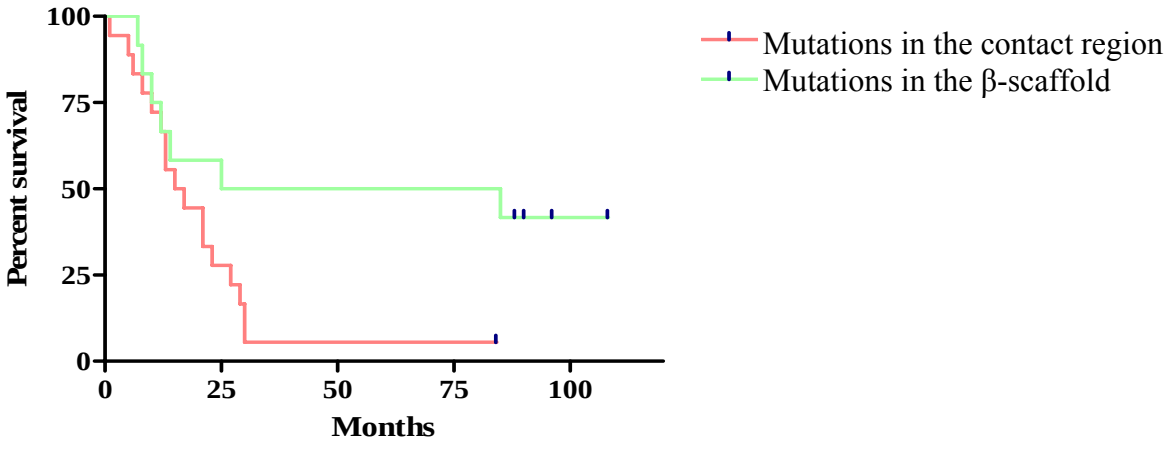
Neither the overall nor the 5 year survival of patients with core domain mutations differed significantly from those without these mutations (p=0.63, p=0.44). Figure 10 represents the Kaplan-Meier curves for 5 year survival.

Figure 10



Inside the core domain, 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). Comparing the survival curves of patients with mutations only on the DNA-binding surface and those with mutations only in the β-sandwich scaffold significant difference was observed (p=0.04) (Figure 11). The median survival was 16 months in the case of "DNA-contact mutation" carriers and 55 months in the case of "β-structure mutation" carriers.

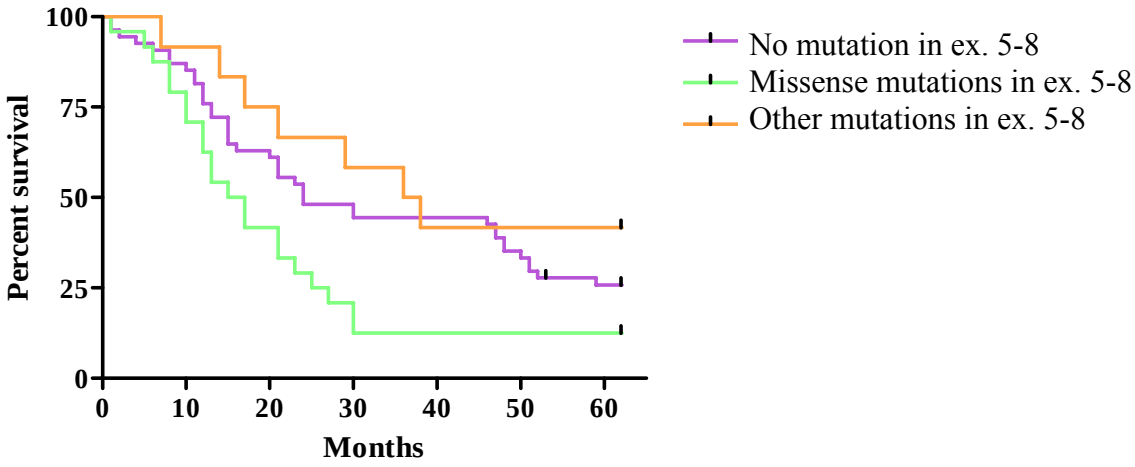
Figure 11



When we examined survival curves considering only the missense mutations there were no significant difference between carriers of the "contact" and the "structural" mutations (p=0.14).

Patients with other than missense mutations (nonsense, splice mutations, deletions) showed significantly longer overall (p=0.012) and 5 year survival (p=0.020) than patients with missense mutations, and did not differ significantly from those without mutations in this region (p=0.331). Five year survival curves of patients with missense and other mutations and patients without core domain mutations can be seen in Figure 12. Interestingly all but one of the four patients with nonsense mutations were still alive 84, 88 and 96 months after the surgery. One patient with nonsense mutation survived 85 months following the surgery.

Figure 12



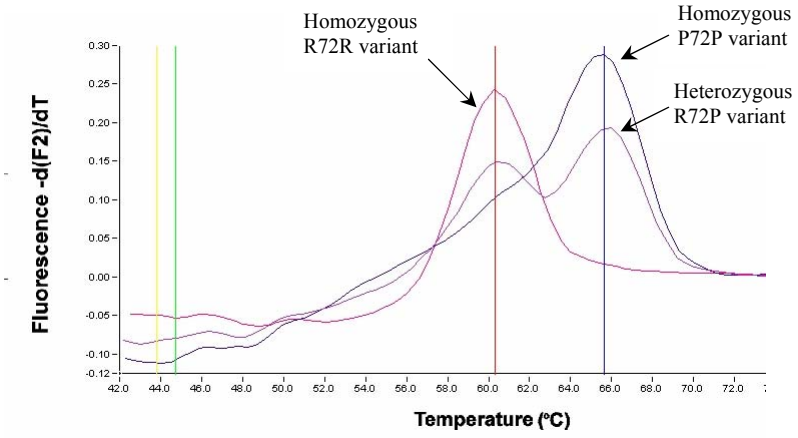
The presence of mutations did not show significant correlation with clinico-pathological parameters, such as tumor size, lymph node metastasis, stage and histological grade. There were no significant differences in the mutation frequencies among the different tumor locations. The missense mutations were the most frequent in oropharynx tumors (85.7%), where these mutations were all found on the DNA-binding surface.

A significant linear trend was seen between tumor stage progression and proportion of subjects with mutations affecting the DNA-binding surface (p=0.022). There was no such a correlation between the tumor stage and the frequency of mutation occurrence in the β-sandwich. In stage 4 tumors the missense mutations were more frequent on the DNA-binding surface than in stage 2-3 tumors (p=0.046).

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. Figure 13 demonstrates representative samples of different genotypes. The allele frequencies were 0.76 R72 and 0.24 P72.

Figure 13



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 median survival was 27 months for the R72R genotypes and 21 months for the R72P genotypes, but the overall survival curves of the two genotypes were not significantly different (p=0.1490). We did not find correlation between the presence of p53 core domain mutation and allele or genotype distribution of codon 72.

Comparing the genotypes of tumor and corresponding normal samples LOH could be detected clearly in 8 cases of the germline heterozygote genotypes. The P72 allele was lost in 5 cases and the R72 allele was lost in 3 cases.

DISCUSSION

Cellular responses to DNA damages have a critical role in tumor genesis. These response mechanisms comprise the cellular processes of DNA damage sensation, cell-cycle arrest, DNA-repair and apoptosis. Genetic aberrations – inherited or acquired – in key regulators of these processes may lead to uncontrolled cell proliferation and accumulation of genetic errors. Once the appropriate combination of mutations accumulates in a given cell type malignant transformation could result. Inherited abnormalities in genes involved in DNA damage response can be expected to alter cancer susceptibility. These inherited gene abnormalities do not generate the transformed phenotype, but set the stage for the genetic changes to occur in the somatic cells which lead to the cellular transformation.

1. Evaluation of genetic alterations in HNPCC suspected patients

The mismatch repair (MMR) system is best known for its role in the post-replicative repair of the errors made by DNA polymerases, that have escaped proofreading. 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.

The identification of germline mutations of either hMSH2 or hMLH1 could be performed in 50-70% of the families that met the Amsterdam criteria for HNPCC, whereas the families not complying with these criteria show a much lower frequency of the MMR gene mutations (65). Since microsatellite instability is found in more than 90% of the HNPCC tumors (66), it is reasonable to test MSI first when screening suspected patients. All of the five patients with identified mutations showed high-level microsatellite instability. We could not detect mutation in two MSI-high patients. It may be due to the limitations of the screening methods, or involvement of genes other than hMLH1 and hMSH2. This is also in line with the findings of the literature, that a significant proportion of HNPCC-like families lack any MMR gene mutations (10). It should be noted that about 15% of sporadic colorectal carcinomas are MSI-high, and they harbor clinical pathologic features similar to those observed in HNPCC, therefore misselection of patients according to less stringent criteria (Bethesda guidelines) can not be excluded.

Four of the seven detected alterations were considered as pathogenic on the bases of published data and due to their predicted deleterious effects on the hMSH2 or hMLH1 protein. These were two nonsense mutations, a large genomic deletion and a splice site mutation leading to frameshift. The splice site mutation c.2210+1G>C (*patient 2*) and the nonsense mutation p.R711X (*patient 4*) in hMSH2 were described earlier as pathogenic mutations (67, 68). The first one causes an out-of frame deletion in exon 13 of the gene leading to a frameshift and probably to a premature stop codon. The latter one leads to premature chain termination in Walker A domain of hMSH2. Loss of immunohistochemical staining in both cases supports the somatic inactivation of the other allele. The nonsense mutation p.E422X in hMSH2 (*patient 1*) and the large deletion g.28756del1787 in hMLH1 (*patient 5*) have not been described previously. The immunohistochemistry of hMSH2 was negative in the case of *patient 1*, suggesting somatic inactivation of the other allele. Whereas, hMLH1 positivity was detected in the case of *patient 5*, that may be due to the other unaffected allele. This large deletion extends from g.26844 (exon 11) to g.28630 (intron 11) resulting in a frameshift which disrupts the interaction domains responsible for heterodimerization with MutS homologues, hPMS2, hMLH3 and hPMS1. There were three missense sequence variants found. Two of them have been identified earlier. The missense mutation p.N127S in *patient 1* is referred by SNP databases www.ncbi.nlm.nih.gov/SNP and http://egp.gs.washingtonedu/data by an allele frequency of 0.02 in healthy populations. The p.V716M mutation found in *patient 2* was identified by Hutter et al. (69) and Cederquist et al. (70) in HNPCC families together with another germline mutation. Cederquist et al (70) found this mutation in a patient with metachronous primary tumors. The allele frequency of this variant was also analyzed (70) and was found to be 0.01 in healthy individuals. The mother of *patient 2* and her sister also carry this mutation in homozygous form without cancer development (Figure 5). Therefore this variant could rather be classified as a rare polymorphism. The missense mutation p.Q48P in *patient 3* results in a change of Gln to Pro in the ATP-ase domain of hMLH1. We have not found reference to this alteration in the literature, and have not received consent to involve other family members in further investigations, therefore we applied different softwares to predict pathogenicity of this variant (71, 72). This substitution took place in a conserved region of hMLH1 shared by diverse classes of ATPases and it was evaluated as possibly damaging (71) and pathological (72) mutation.

Patient 1 and *patient 2* both had two germline mutations. In *patient 1* p.E422X accompanied by p.N127S in hMSH2, in *patient 2* c.2210+1G>C in hMSH2 associated with p.V716M in hMLH1 were identified respectively. The pedigree analysis supported that the nonsense mutation in the first case and the c.2210+1G>C mutation resulting in defective splicing in the other case were essential in cancer development. The missense mutations accompanying the above alterations did not cause pathogenicity when occurred alone in family members (Figures 4 and 5). The presence of these polymorphisms together with the pathogenic mutations caused an early onset of tumor at the age of the twenties and early thirties of the patients, whereas relatives carrying the single pathogenic mutations developed cancer in their forties and fifties (Table 2, 3) or have not had disease yet. It is of interest that earlier studies (69, 70) found p.V716M polymorphism associated with other pathogenic mutations in HNPCC patients, furthermore Cederquist et al found this alteration in a patient with double primary metachronous tumors. In our study *patient 2* had double synchronous colorectal carcinoma too. The paternal grandfather of *patient 2* developed colorectal cancer over the age of 80, but he carried none of the alterations defined above. These facts suggest that his colorectal cancer was a sporadic disease.

These findings demonstrate that causative mutations coupled with single nucleotide polymorphisms have worse prognostic values, such as earlier (possibly multiplex) tumor manifestation, and suggest close follow-up of carriers from their mid-twenties.

Our results suggest that with the use of Bethesda guidelines as criteria to perform MSI testing, several HNPCC patients can be identified who would be missed by application of the more stringent Amsterdam I and II criteria.

Identification of HNPCC patients has a therapeutic relevance too. Unfortunately cells defective in MMR are relatively resistant to 5-fluorouracil (5-FU), the most commonly employed chemotherapeutic agent for colorectal cancer (21). Consequently, HNPCC patients do not benefit from 5-FU chemotherapy. MMR proteins have also been implicated in recombination and double strand break repair (73), which may explain the sensitivity of MMR-deficient cells to ionizing radiation or topoisomerase inhibitors (74,22). Recent studies support the hypothesis that inhibition of COX-2 enzyme by nonsteroidal anti-inflammatory drugs may provide a mechanism for cancer prevention in HNPCC patients (75).

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

MMR components have been shown to participate in the recognition of DNA adducts caused also by platinum-based drugs used in cancer chemotherapy (19). The recognition of the damaged bases by MMR initiates a signal transduction pathway that can activate cell-cycle checkpoint and trigger apoptosis both in a p53-dependent and p53-independent manner (76). Among cancers sensitive to cisplatin the greatest response can be achieved in seminomatous germ cell tumors, whereas mature teratomas are clinically resistant to the effects of chemotherapy. Owing to their diverse histological development and the differences seen in response to chemotherapy, with high cure rates in most patients but resistance in a small percentage of cases, testicular germ cell tumors offer an attractive model to examine the mechanisms of chemosensitivity and resistance of tumor cells to platinum derivatives.

Losses or defects of MMR components can confer resistance to cisplatin (20, 77). Several studies demonstrated correlation between cisplatin resistance and MMR deficiency in certain carcinoma cell lines (78, 79). Nevertheless, some recent studies found no association between treatment response and MSI status in ovarian and cervical cancer (80, 81).

Earlier studies did not find notable microsatellite instability in testicular germ-cell tumors (82, 83). In contrast, Mayer et al. found that 45% of TGCT specimens with refractory disease had unstable microsatellites (27). The majority of MSI cases are caused by hMLH1 gene promoter hypermethylation in sporadic colorectal (84), gastric (85) and endometrial carcinomas (86). Koul et al. found hMLH1 hypermethylation in 6.5% of GCT cases (87). They showed that methylation of the promoter region between -269 and -169 from the transcriptional start was associated with absent or downregulated hMLH1 expression. We found hMLH1 methylation in 21.6% of cases in the distal promoter region between -622 and -575. hMLH1 methylation correlated well with loss of hMLH1 expression ($p<0.0001$). We detected 2 cases with loss of hMLH1 protein expression that showed no hMLH1 methylation. This suggests that hMLH1 deficiency can be caused by mechanisms other than hypermethylation of the hMLH1 gene, (e.g. allelic loss, mutations). In three cases strong hMLH1 protein expression was detected with hMLH1 methylation. This may be explained by the heterogenity of the tumor tissue where immunohistochemistry represents a tissue section, while methylation could be detected in a heterogeneous cell population. Loss of hMSH6 protein expression occurred in 4 cases, in 3 of them, loss of hMSH2 expression was observed

as well. Moreover, separate loss of hMSH2 expression was not observed. This is in line with the finding that hMSH2 and hMSH6 exist as heterodimers (88).

Likewise Mayer et al. (27) we found no correlation between MSI and immunohistochemical assessment of hMLH1, hMSH2 and hMSH6. In addition, we also found no relationship between hMLH1 methylation and the MSI of tumor samples. Tumors that exhibit microsatellite instability are frequently designated as RER^{+}, indicating that the mutations are generated by replication errors; however the source of mutations has not been clearly established. For example, heteroduplex DNA yielding nonparental microsatellite alleles could arise not only from errors in DNA synthesis, but also from recombination intermediates. Microsatellite instability may therefore be imagined to be caused by mutations in DNA polymerases or recombination proteins. Liu et al. (89) reported that the mutation in nine of ten cases of sporadic colon cancers which exhibited microsaellite instability was not one of those reported to be associated with mismatch repair.

Neither MMR deficiency nor MSI showed significant correlation with clinico-pathological features, such as tumor stage, histology or chemoresistance.

Our results suggest that, in contrast to colorectal cancer and other solid tumors (84, 85, 86), hMLH1 promoter methylation and MMR (hMLH1, hMSH2, hMSH6) protein deficiency do not contribute to the MSI status of GCTs. In their study Claij et al. (90) point out that in many cases the extent of microsatellite instability was not as dramatic as found in HNPCC-related tumors and the underlying genetic defect is unclear. Therefore, while the mismatch repair status of tumors may become an important determinant in the choice of chemotherapeutic intervention, the significance of MSI in sporadic cancer remains elusive.

Although CpG island methylation of hMLH1 is a major mechanism of inactivation of MMR, it proceeds gradually. Multiple loci can become simultaneously methylated in the drug-resistant cells and hMLH1 may be only one of several genes whose inactivation can influence drug sensitivity. Branch et al showed a relatively minor contribution of defective mismatch repair to cisplatin resistance in contrast to abrogated p53 response (91).

Our results support that cisplatin resistance of a small proportion of testicular tumors seems to be determined by multiple factors rather than a uniform mechanism such as mismatch repair deficiency. Recent studies have revealed that such factors as reduced drug uptake (92), overexpression of drug exporters as LRP (93, 94) and MRP2 or the thiol-conjugating enzyme GSTπ (93) can confer resistance at least in some cases. P53 mutations, albeit at a very low frequency have been found in male GCTs and may represent one molecular means by which

cisplatin resistance is acquired (95). Juric et al. have shown a potential role of EGR1 in p21-induced cell cycle arrest and intrinsic chemotherapy resistance of mature teratomas (96). Comparative genomic hybridization (CGH) studies have revealed that chromosomal amplification at multiple sites is associated with cisplatin resistance (97).
However, a uniform explanation is still missing, it is most likely, that chemotherapy-resistant phenotype is related to somatic differentiation. Gene expression profiling in the future may help to define chemoresitance-specific signature of TGCTs.

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

Acute DNA damage triggers a rapid p53 response that starts with p53 accumulation, p53 post-translational modifications and culminates with either apoptosis or growth arrest after triggering transcription-dependent and/or –independent pathways. It is generally accepted that the apoptotic activity of p53 is the main target of p53 gene mutations (98). The absence of the apoptotic activity could therefore account not only for tumor progression, but could also explain treatment resistance phenomena.

Our mutational analysis covered the gene region encoding the core domain of p53, since the 80% of p53 mutations is associated with the DNA-binding activity of this domain. The most frequent changes at DNA level were C:G>T:A and A:T>G:C transitions (57.89%). The deamination of cytosine leads to the C:G>T:A and the deamination of adenine leads to the A:T>G:C transition, which reflects the mutagenic effects of exogenous carcinogens. 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%). It could be explained by the higher deamination rate of cytosines found at CpG dinucleotides along the p53 gene. It has been reported that all of the CpGs are methylated in p53 gene. It has been assumed that the deamination of 5-methyl-cytosines leading to T:G mismatches that are not efficiently repaired (98) could cause this high transition rate. It has been demonstrated that such a potent carcinogen as benzo(a)-pyrene diol epoxide (BPDE) have a higher affinity for methylated CpGs than their unmethylated counterparts (99). BPDE is the main metabolite of benzo(a)-pyrene, which is present in high quantity in tobacco smoke (99). It is interesting, albeit CpG dinucleotides are near equally distributed between the coding regions of the β-scaffold and the DNA-binding surface, the CpG targets for hot spot mutations all can be found in the sequence encoding the DNA-binding surface. We found six C:G>T:A transitions at CpG sites, and five of them were located in codons of the DNA-

binding surface. It can be supposed that these CpG site mutations affecting the DNA-binding surface have some selection advantage against those affecting the β-structure. The transition of A:T>G:C was found to be more frequent among the point mutations of the β-structure (41.67%) than those of the DNA-binding surface (10.52%). Although the difference was not significant (p=0.078), it should be noted that the A:T>G:C transitions were more frequent in our sample group (18.42%) compared to the value given by the IARC TP53 Mutation Database for HNSCC (9.7%) (R11 release) (100).

Although the majority of the alterations we found were missense mutations (67.6%), considerable amounts of nonsense mutations (10.8%), splice mutations (5.4%) and deletions (10.8%) were also found. Except for the deletions and the TA>AT inversion all the mutations we detected can be found in the IARC TP53 Mutation Database (100). However, we could not show general correlation between the presence of p53 mutation and the prognosis, significant differences were observed in the impact of the mutations depending on their type and location. An enormous body of data has provided evidence that the majority of missense mutations are found in the core domain of the p53 protein. It is noteworthy that mutations, such as nonsense mutations, deletions and insertions that lead to either the synthesis of a truncated protein or to the complete loss of a protein, are more frequent outside the core domain, in exons 4, 9 and 10. Inside the core domain we have shown a similar tendency, that is missense mutations were significantly more frequent on the DNA-binding surface, while other types of mutations (nonsense, splice mutations, deletions) occurred more often in the β-sandwich (p=0.03). Several studies demonstrated that p53 mutations were associated with bad prognosis, furthermore, mutations at residues involved in DNA contacts had even worse prognosis (101). We also found that patients with mutations within the DNA-binding residues had poorer survival than those carrying mutations in the β-sheets (p=0.04). A recent study (102) on breast cancer patients has found that missense mutations located within the DNA-binding motifs had a similar prognostic value as non-missense mutations. All the same we found that patients with other than missense mutations (nonsense, splice mutations, deletions) showed significantly longer survival (p=0.012) than patients with missense mutations. Moreover, the carriers of nonsense mutations were observed to survive for the longest periods. Although the patients with β-structure mutations survived longer compared to the DNA-contact mutation carriers, the difference had dimmed when solely the missense mutations were considered. These results suggest that missense mutations, which are dominantly found on the DNA-contacting surface, determine a poorer outcome. It may be

supposed that missense mutations are responsible for the oncogenic activity of mutant p53, while deletions, insertions and nonsense mutations, which are characteristic of tumor suppressor genes, mainly contribute to the loss of function properties. Nonsense and frameshift mutations may render a less progressive phenotype to the mutant tumor cells. Nevertheless, survival advantage of patients with nonsense mutation versus patients without core domain mutation is not clear yet. However, conflicting results are available for several tumor types, Tomizawa et al. (103) have showed as well that non-small-cell lung cancer patients carrying missense mutations had a poorer prognosis compared with patients who had null mutations.

We have not found a significant correlation between the presence of mutations and tumor stages. It may be due to that p53 mutations occur at an early stage, whereas our patient group comprised advanced cases. The significant trend 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 progressive phenotype.

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.

A subset of p53 mutants has been shown to bind and inhibit p73, a homolog of p53 that can induce apoptosis in p53-deficient cells (43). The binding of such mutants is influenced by the common Arg/Pro polymorphism at position 72 of the p53 protein. Marin et al. showed (43) that R72-containing mutant p53 protein was a more potent inhibitor of p73-induced apoptosis.

Analyzing codon 72 polymorphisms in 89 HNSCC patients and 216 healthy individuals no significant difference was seen between the allele distributions. Core domain mutations were of similar frequencies in R/R homozygotes and R/P heterozygotes. The survival curves and the tumor stages of the homo- and the heterozygotes were not significantly different. The inhibition of p73 confers also to apoptosis induced by chemotherapeutic drugs. Bergamaschi et al. have provided an evidence that clinical response to chemo- and radiotherapy in advanced head and neck cancer is influenced both by the properties of the p53 mutants and the allelic variant at codon 72 (41). The response has been found to be less favorable when the mutant occurred in the R72 rather than P72 form (41). Considering the above results, we can conclude that the codon 72 genotype alone has no predictive value for clinical outcome, but allelotyping of the mutant allele may be useful to predict chemotherapeutic sensitivity.

Our results suggest an idea of dual character of p53 gene in which missense mutations render an oncogenic property to the protein, while other types of mutations disrupt its tumor suppressor functions. According to the conception of Petitjean et al. loss of transactivation activities, and to a lesser extent, dominant negative activities are the main driving forces that determine p53 mutation patterns (101). In contrast we suppose that oncogenic (gain of function) properties provide a selection advantage to missense mutants. This assumption can be supported by the following observations.

- The majority of mutations are missense mutations in the core domain which alter the DNA-binding specificity and transactivation properties of the p53 protein without affecting the transactivation and tetramerization domains. Loss of transactivation activities may be the consequence of null mutations as well, but the survival of patients with these mutations is significantly better.
- CpG site hot spot mutations are exclusively located on the DNA-contacting surface.
- A significant trend was observed between the tumor stage progression and the frequency of missense mutations on the DNA-binding surface supporting the assumption that these mutations are responsible for a more aggressive phenotype.
- The R72 variants of mutant p53 are more potent inhibitors of p73 that leads to a selective advantage against the P72 mutants.

Mutant p53 partly or entirely loses its binding affinity to the specific DNA-target sequences of wild type p53. Several mutants gain ability to influence the transcription of new target genes via protein-protein interactions with transcription factors, coactivators or repressors resulting in a “change in spectrum” phenotype (104). The large diversity of p53 mutants can be translated into a wide spectrum of distinct phenotypes. Each p53 mutant is a unique protein with a specific behavior in respect of its DNA-binding activity, target gene spectrum (105), and its effects on chemoresistance (106, 107).

Altogether these findings strongly indicate that missense mutant p53 proteins do not represent the mere loss of wild-type p53 activities, but gain new additional oncogenic functions which contribute to the development, maintenance, spreading and resistance to anti-cancer treatment of the tumor.

SUMMARY

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 the mediators of these processes may lead to uncontrolled cell proliferation and accumulation of genetic errors which may eventually result in tumor development. Exposure to environmental DNA damaging conditions could result not only in genetic alterations, but also in modifications of the chromatin structure, changing the transcriptional activity of several genes. Such an epigenetic alteration is methylation of 5' cytosines of CpG islands in promoters of tumor suppressor genes. A synergy between genetic and epigenetic alterations drives the tumor progression.

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 mismatch repair 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 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.

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. 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.

It was shown earlier that cells defective in MMR are relatively resistant to fluoropirimidines 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.

P53 as a “master regulator” of cell-cycle, DNA repair and apoptosis has a critical role in DNA-damage response. Inactivating mutations in p53 gene are the most common genetic alterations in human cancer. Analysis of aberrations in the p53 gene has diagnostic and prognostic utility in head and neck squamous cell carcinoma (HNSCC). The majority of the alterations we found in the core domain encoding region were missense mutations, whereas notable proportions of nonsense mutations, splice site mutations and deletions were also found. Most of the point mutations were C:G>T:A transitions as a consequence that methylated cytosines in CpG dinucleotides are more sensitive to deamination than their unmethylated counterparts. Five out of six C:G>T:A transitions at CpG sites were found in codons of the DNA-binding surface, although CpG dinucleotides are almost equally distributed between the coding regions of the β-scaffold and 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. Inside the core domain the missense mutations were significantly more frequent on the DNA-binding surface, while other types of mutations (nonsense, splice mutations, deletions) occurred more often in the β-sandwich.

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, whereas our patient group comprised advanced cases. All the same 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. We found that 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.

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.

Comparing the germline allele and genotype distribution of R72P polymorphism of p53 there was no significant difference seen between the HNSCC patients and a healthy control population. Core domain mutations were of similar frequencies in R/R homozygotes and R/P heterozygotes. The survival curves and the tumor stages of the homo- and the heterozygotes were not significantly different. All the same, it was shown earlier that chemo- and radiotherapeutic response is less favorable when p53 mutation occurs on the R72 rather than the P72 allele. Considering the above results, 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 the therapeutic sensitivity.

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ANNEXES

ANNEX 1

Characterization of the patients

1. HNPCC suspected patients referred in *Materials and methods 1.1.1.* and in *Results 1.2.*

Amsterdam criteria	***patient 1***	***patient 2***
At least three relatives with CRC (Amsterdam I)	+	-
Three or more relatives with HNPCC-associated cancer (CRC, endometrium, small bowel, ureter or renal pelvis) (Amsterdam II)	+	-
One affected patient is a first-degree relative of the other two	-	-
Two or more successive generations affected	+	-
Cancer in one ore more affected relatives diagnosed at age < 50 y	+	+
Familial adenomatous polyposis excluded	+	+
Tumors verified by pathologic examination	+	+
Bethesda guidelines	***patient 1***	***patient 2***
Individuals with cancer in families that meet the Amsterdam criteria	-	-
Individuals with two HNPCC-related tumors, including synchronous and metachronous CRC or associated extracolonic cancer	-	+
Individuals with CRC or endometrial cancer diagnosed at age < 45 y	+	+
Individuals with CRC and a first-degree relative with CRC or HNPCC-related extracolonic cancer diagnosed at age < 45 y, or with colorectal adenoma diagnosed at age < 40 y	+	+
Individuals with right-sided CRC with undifferentiated histopathology diagnosed at age < 45 y	+	+
Individuals with signet-ring-cell-type CRC diagnosed at age < 45 y	-	+
Individuals with adenomas diagnosed at age < 40 y	-	-

2. TGCT patients' data referred in *Materials and methods 1.1.2.*

	Patients	Histology	Stage	Age (years)
Chemoresistant	36	S	IIIB	34
	49	Cc+Ec+T	IIIB	23
	51	Cc+Ec+T	IIIB	25
	58	T	IIIB	30
	61	Ec+T	IIIB	24
	108	S+T	IIIB	38
	171	Ec+Ys	IIIB	46
	172	Ec+T	IIC	19
	193	T	IIIB	31
	199	Ec+T	IIIB	31
	207	Ec+S+T	IIIB	48
	214	Ec+S+T	IIIB	29
	220	Ec+T	IIC	34
	222	Ec+T	IIIB	26
	249	S	IIIB	33
Chemosensitive	30	Ec+Ys	IB	34
	47	Ec+S+T	IA	40
	60	T	IIIB	17
	76	Cc+Ec+T	IIIA	17
	78	T	IB	39
	85	Cc	IIIB	23
	91	T	IIC	19
	93	S	IB	34
	98	Ec+S	IIA	33
	99	S+T	IB	40
	104	S	IB	37
	105	Ec+T	IIIA	46
	114	Ec+S	IB	40
	116	Ec+S+T	IIA	35
	117	Cc+Ec	IS	41
	119	Ec+T	IIIA	33
	129	Ec+T	IB	21
	131	S	IA	31
	132	S	IB	47
	142	Ec+S	IB	26
	143	S	IA	43
	145	Ec	IIIA	27
	146	Cc+Ec+T	IIA	20
	150	Ec	IIA	25
	151	Ec+Ys	IIIA	31
	154	Cc+Ec+T	IB	24
	157	S	IB	52
	158	S	IB	60
	161	Ec+T	IIIA	20
	165	Ec	IIB	22
	166	Ec+Ys	IS	25
	167	Ec+S	IB	44
	168	Ec+T	IIIA	17
	173	Ec+S	IA	38
	176	Ec+T	IIB	26
	187	S	IA	45

Cc: Choriocarcinoma; Ec: Embryonal carcinoma; S: Seminoma; T: Teratoma; Ys: Yolk sac tumor

3. Data of the HNSCC patients referred in *Materials and methods 2.1. and* in *Results 3.1.*

Site	Patients	T	N	S	Grade	Age at diagnosis (years)	Survival (months)
Oral cavity	4	3	0	3	1	53	21
	22	2	2	4	1	48	82
	42	3	0	3	1	68	14
	52	2	0	2	1	70	12
	60	4	2	4	1	60	13
	72	2	0	2	1	63	30
	110	3	2	4	1	44	88*
	125	2	1	3	1	61	1
	9	2	0	2	2	72	36
	18	3	2	4	2	59	12
	31	3	0	3	2	57	12
	49	2	0	2	2	59	23
	50	2	0	2	2	57	96*
	56	3	2	4	2	72	98*
	57	4	1	4	2	48	20
	59	3	2	4	2	62	15
	79	4	1	4	2	48	51
	83	2	2	4	2	66	13
	85	2	0	2	2	39	12
	90	2	1	3	2	52	8
	109	3	1	3	2	39	71
	120	4	2	4	2	56	5
	123	2	0	2	2	47	48
	124	3	0	3	2	58	59
	126	3	0	3	2	38	15
	131	2	0	2	2	52	25
	133	3	1	3	2	71	38
	61	2	1	3	2	55	96*
	16	4	2	4	3	42	10
	33	3	0	3	3	47	15
	97	3	0	3	3	64	47
	101	4	0	4	3	62	71
	108	4	3	4	3	40	24
	116	2	0	2	3	49	ND
Oropharynx	27	4	0	4	1	46	52
	127	4	2	4	1	45	30
	38	3	1	3	2	61	50
	41	2	2	4	2	54	8
	148	4	2	4	2	57	46
	70	3	1	3	2	55	27
	105	4	1	4	2	61	6
	114	3	2	4	2	42	13
	119	4	2	4	2	47	15
	128	2	2	4	2	61	1
	135	4	0	4	2	48	10
	39	4	1	4	3	44	11
	73	2	0	2	3	38	78
	92	1	1	3	3	59	108*
	113	2	2	4	3	42	16

Site	Patients	T	N	S	Grade	Age at diagnosis (years)	Survival (months)
Hypopharynx	19	2	0	2	1	46	108*
	62	4	0	4	1	74	8
	82	3	1	3	2	64	82
	3	3	2	4	2	45	6
	8	3	1	3	2	50	47
	25	3	1	3	2	48	21
	54	3	1	3	2	44	4
	84	4	2	4	2	44	74
	98	4	2	4	2	49	1
	112	3	0	3	2	46	29
	134	3	0	3	2	67	48
	5	3	2	4	3	45	24
	43	1	2	4	3	57	96*
	51	4	1	4	3	56	89*
	55	3	2	4	3	47	15
	104	3	3	4	3	44	7
	106	4	2	4	3	45	17
	117	3	2	4	3	56	8
	121	2	2	4	3	45	30
	129	3	2	4	3	39	117
	143	4	2	4	3	52	21
	150	3	2	4	3	74	30
	6	2	0	2	3	52	51
Larynx	44	1	0	1	1	49	24
	76	3	2	4	1	59	2
	87	3	0	3	1	46	90*
	32	3	0	3	2	65	12
	34	3	0	3	2	46	21
	58	4	0	4	2	63	23
	80	3	2	4	2	70	11
	81	3	2	4	2	50	13
	95	3	2	4	2	58	74
	107	3	0	3	2	59	89*
	122	3	0	3	2	47	62
	149	3	1	3	2	49	84*
	12	3	0	3	3	65	85
	21	4	0	4	3	42	10
	37	4	0	4	3	54	53*
	71	4	2	4	3	48	21
	94	4	0	4	3	52	17

T-N-S: tumor size–lymph node metastases-stage according to the UICC classification

***** alive at the time of the investigation

ND: not detected

ANNEX 2

Primers applied in microsatellite analyses

Primers used in microsatellite instability analysis described in *Materials and Methods 1.4.*

Bat-25:
sense: 5'-HEX-TCG CCT CCA AGA ATG TAA GT-3'
antisense: 5'-TCT GCA TTT TAA CTA TGG CTC-3'

Bat-26:
sense: 5'-NED-TGA CTA CTT TTG ACT TCA GCC-3'
antisense: 5'-AAC CAT TCA ACA TTT TTA ACC C-3'

D2S123:
sense: 5'-FAM-AAA CAG GAT GCC TGC CTT TA-3'
antisense: 5'-GGA CTT TCC ACC TAT GGG AC-3'

D5S346:
sense: 5'-FAM-ACT CAC TCT AGT GAT AAA TCG GG-3'
antisense: 5'-AGC AGA TAA GAC AGT ATT ACT AGT T-3'

D17S250:
sense: 5'-NED-GGA AGA ATC AAA TAG ACA AT-3'
antisense: 5'-GCT GGC CAT ATA TAT ATT TAA ACC-3'

ANNEX 3

Primers applied in sequencing analyses

1. Primers used in the amplification and sequencing reactions of hMLH1 and hMSH2 genes desdribed in *Materials and Methods 1.5.*

   hMLH1:

   Exon 1: sense: 5'-GAC GTT TCC TTG GCT CTT CTG-3'
   antisense: 5'-CCG TTA AGT CGT TAG CCC TTA AGT-3'

   Exon 2: sense: 5'-TAT TTT CTG TTT GAT TTG CCA G-3'
   antisense: 5'-TGA CTC TTC CAT GAA GCG C-3'

   Exon 3: sense: 5'-TGG AAA AAT GAG TAA CAT GAT-3'
   antisense: 5'-CAC AGG AGG ATA TTT TAC ACA-3'

   Exon 4: sense: 5'-CCC AGC AGT GAG TTT TTC TTT-3'
   antisense: 5'-GAT TAC TCT GAG ACC TAG GC-3'

   Exon 5: sense: 5'-GAT TTT CTC TTT TCC CCT TGG G-3'
   antisense: 5'-CAA ACA AAG CTT CAA CAA TTT AC-3'

   Exon 6: sense: 5'-TTG CCA GGA CCA TCT TGG G-3'
   antisense: 5'-ACT CCC AGA TTT TGG ACT GT-3'

   Exon 7: sense: 5'-CTA GTG TGT GTT TTT GGC-3'
   antisense: 5'-CAT AAC CTT ATC TCC ACC-3'

   Exon 8: sense: 5'-AAA TCC TTG TGT CTT CTG CTG-3'
   antisense: 5'-GTG ATG GAA TGA TAA ACC AAG-3'

   Exon 9: sense: 5'-GCT TCA GAA TCT CTT TTC TA-3'
   antisense: 5'-GTG GAT TTC CCA TGT GGT TC-3'

   Exon 10: sense: 5'-GGA CAG TTT TGA ACT GGT TGC-3'
   antisense: 5'-GAG GAG AGC CTG ATA GAA CAT CTG-3'

   Exon 11: sense: 5'-TCT AAG GTA ATT GTT CTC TCT TA-3'
   antisense: 5'-AAG TAG CTG GAT GAG AAG CG-3'

   Exon 12: sense: 5'-TTA ATA CAG ACT TTG CTA CCA G-3'
   antisense: 5'-GGT AGG CTG TAC TTT TCC C-3'

   Exon 13: sense: 5'-GGT TCA TTC ACA GCT CTG TAG-3'
   antisense: 5'-CAA CAT GAC TGC TTT CTC CA -3'

   Exon 14: sense: 5'-AAG TGG GGT TGG TAG GAT TC-3'
   antisense: 5'-CTC TGC TTG TTC ACA CAC TC-3'

   Exon 15: sense: 5'-AAT TCA GCT TTT CCT TAA AGT C-3'
   antisense: 5'-ACT ATT TTC AGA AAC GAT CAG TT -3'

   Exon 16: sense: 5'-TTC ATG TTC TTG CTT CTT CC-3'
   antisense: 5'-GAA GTA TAA GAA TGG CTG TC-3'

Exon 17: sense: 5’-TGT CCT TTT TCC TGC AAG C-3’
antisense: 5’-TTT CCC TCC AGC ACA CAT G-3’

Exon 18: sense: 5’-GAG GTA TTG AAT TTC TTT GGA C-3’
antisense: 5’-GTG TGC ATC ACC ACT GTA CC-3’

Exon 19: sense: 5’-CAA ACA GGG AGG CTT ATG AC-3’
antisense: 5’-AAG AAC ACA TCC CAC AGT GC-3’

hMSH2:

Exon 1: sense: 5’-CTT CAA CCA GGA GGT GAG GAG GT-3’
Antisense: 5’-GAA AGG AGC CGC GCC ACA AG-3’

Exon 2: sense: 5’-ATG TAA TAT CTC AAA TCT GTA ATG T-3’
antisense: 5’-ATA AGT AAA TTA AAA AGG AAG ATA A-3’

Exon 3: sense: 5’-TGT TCA AGA GTT TGT TAA ATT TTT-3’
antisense: 5’-TGG AAT CTC CTC TAT CAC TAG ACT-3’

Exon 4: sense: 5’-TCT TAT TCC TTT TCT CAT AGT AG-3’
antisense: 5’-TAT TGT AAT TCA CAT TTA TAA TCC-3’

Exon 5: sense: 5’-AGT GGT ATA GAA ATC TTC-3’
antisense. 5’-ACC AAT CAA CAT TTT TAA CCC-3’

Exon 6: sense: 5’-TTT CAC TAA TGA GCT TGC C-3’
antisense: 5’-CAG GTT ACA TAA AAC TAA CG-3’

Exon 7: sense: 5’-TGA GAC TTA CGT GCT TAG TTG-3’
antisense: 5’-TGT ATG AGT TGA AGG AAA ACA-3’

Exon 8: sense: 5’-AAT GAG ATC TTT TTA TTT GTT TGT T-3’
antisense: 5’-ACT GCT TAA ATT AAA AAA GTA TAT TG-3’

Exon 9: sense: 5’-AGG ATT TTG TCA CTT TGT TCT G-3’
antisense: 5’-CAA AAG AAT TAT TCC AAC CTC-3’

Exon 10: sense: 5’-GTA GTA GGT ATT TAT GGA ATA C-3’
antisense: 5’-GGA ATT AAT AAA GGG TTA A-3’

Exon 11: sense: 5’-TTA ATA AAA CTG TTA TTT CCG ATT TG-3’
antisense: 5’-AGC CAG GTG ACA TTC AGA ACA TT AT-3’

Exon 12: sense: 5’-CAG TAT TCC TGT GTA CAT TTT CTG T-3’
antisense: 5’-AAG CCC AAA AAC CAG GTT T-3’

Exon 13: sense: 5’-AAT CTT GCT TTC TGA TAT AAT TTG-3’
antisense: 5’-CAT TTC TAT CTT CAA GGG ACT AGG A-3’

Exon 14: sense: 5’-TCA TGT AAT TAT GTG CTT CAG-3’
antisense: 5’-GTA CTC CAA TAG TAC ATA CC-3’

Exon 15: sense: 5’-TGT CTC TTC TCA TGC TGT CC-3’
antisense: 5’-TAA GTT AAA CTA TGA AAA CAA ACT G-3’

Exon 16: sense: 5’-TTA CTA ATG GGA CAT TCA CAT G-3’
antisense: 5’-TAC CTT CAT TCC ATT ACT GGG-3’

2. Primers designed for the amplification and sequencing of the exons 5-9 of p53 gene referred in *Materials and Methods 2.3.*

   p53 exons 5-6:

   sense: 5'-GTT TCT TTG CTG CCG TGT TCC-3'
   antisense: 5'-TTG CAC ATC TCA TGG GGT TAT-3'

   p53 exon 7:

   sense: 5'-ATC TTG GGC CTG TGT TAT CTC C-3'
   antisense: 5'-CAG GGT GGC AAG TGG CTC-3'

   p53 exons 8-9:

   sense: 5'-CCT ATC CTG AGT AGT GGT AAT-3'
   antisense: 5'-CAA GAC TTA GTA CCT GAA GGG TGA-3'

ANNEX 4

TaqMan assays applied in promoter methylation analyses

Primers and TaqMan probes for referred in *Materials and Methods 1.6.:*

hMLH1:

forward: 5'-CGT TAT ATA TCG TTC GTA GTA TTC GTG TTT-3'
reverse: 5'-CTA TCG CCG CCT CAT CGT-3'
TaqMan probe: 5'-6FAM-CGC GAC GTC AAA CGC CAC TAC G-TAMRA-3'

ACTB:

forward: 5'- TGG TGA TGG AGG AGG TTT AGT AAG T-3'
reverse: 5'- AAC CAA TAA AAC CTA CTC CTC CCT TAA-3'
TaqMan probe: 5'-6FAM-ACCACCACCCAACACACAATAACAAACACA-TAMRA-3'

ANNEX 5

Primers and hybridization probes applied in codon 72 polymorphism analysis of p53 gene

Primers and probes referred in *Materials and Methods 2.4.*

sense: 5'-GAT GCT GTC CCC GGA CGA-3'
antisense: 5'-AGG GGC CGC CGG TGT AG-3'

anchor: 5'-CCA GAT GAA GCT CCC AGA ATG CCA GAG GCT-FL-3'
sensor: 5'-LC Red640-TCC CCC CGT IIC CCC TGC ACC-PH-3'