SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Studying of Ki-67, FLT3 and JAK2 mutations in tumor cell-lines and in samples of patients with colorectal carcinomas

by Tamás Bubán, M.D.

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Inset of the doctoral dissertation

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The Examination takes place at the Library of the Institute for Pathology, Medical and Health Science Center, University of Debrecen
30th November, 2012.

Head of the **Defense Committee**:	Zoltán Nemes, M.D., D.Sc.
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The Ph.D. Defense takes place at the Lecture Hall of the Department of Urology, Medical and Health Science Center, University of Debrecen
30th November, 2012.

1. INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 The most important clinical and pathological characteristics of the colorectal carcinomas

Colorectal carcinoma (CRC) is the third most frequent tumor in women and the fourth in men worldwide and in accordance with this it is the second most frequent cause of cancer mortality in the majority of the developed countries. Incidence of CRC is highest in the most civilized and lowest in the developing countries with a tenfold difference between them. One of the most important risk factors is age as the incidence rises sharply between the age of 40 and 50. The probability of the development of CRC in a population with average risk is about 5% during the expected lifetime. Histologically these tumors are mainly adenocarcinomas. Prognosis and the further treatment are basically determined by the tumor's TNM stage. CRC can be divided into sporadic, familial and hereditary groups.

1.2 Sporadic CRC

In the case of sporadic CRC, which is 70% of the CRC cases, there is no colon tumor in the family history of the patient. Cancer develops usually over the age of 50. Besides dietary and environmental factors several mutations in the somatic cells can lead to development of the cancer. If the patient has a history of removal of a colon polyp larger than 1 cm the relative risk rises to 3.5-6.5. If an operation was performed because of CRC a new primer (metachronous) tumor can develop in the 1.5-3.0% of the patients.

1.3 Familial CRC

About 25% of the patients have a positive familial history of CRC but none of the hereditary syndromes can be verified. The risk of CRC is increased in these cases but not so high than that in the hereditary disorders. Inheritance of

familial CRC is probably polygenic. Accumulation of less well-known hereditary genetic alterations with lower effects can be in the background.

1.4 Hereditary CRC

In less than 10% of all patients with CRC we can verify a hereditary predisposition to develop the disease for which a special germ cell mutation is responsible. We can divide this group into two parts on the basis of the presence of polyps. Disorders accompanied with polyposis involve syndrome of familial adenomatous polyposis (FAP and MUTYH associated polyposis) and syndromes of hamartomatous polyposis (e.g. syndrome of Peutz-Jeghers). Disorders not accompanied with polyposis include syndromes of Lynch, familial CRC type X and other rare disorders. These malformations have a really high risk for development of CRC. Hereditary mutations can be identified and used for screening.

1.4.1 Lynch syndrome

Lynch syndrome is the most frequent disease among hereditary CRC and it represents 1-4% of all CRC cases. This disorder can be characterized by early development of CRC and other (e.g. endometrial, ovarial, stomach, urinary tract) adenocarcinomas. It is autosomal dominantly inherited. Alterations of genes repairing mistakes of DNA replication (mismatch repair – MMR genes, most often hMSH2 and hMLH1) play a role in the development of the disease. Because of their diminished activity mistakes accumulate in the DNA mainly in the microsatellites. If it occurs in the genes regulating proliferation a tumor can develop.

Clinical (Amsterdam and Bethesda) criteria were defined to facilitate the clinical diagnosis of the HNPCC. Immunohistochemical (IHC) and microsatellite instability examination (MSI) are useful to be performed in tumor samples of patients selected on the basis of these criteria. If Lynch syndrome is

suspicious according to these examinations sequencing of the MMR genes and other additional tests can be performed (studies of large deletions with MLPA, examination of hypermethylation, and analysis of 3' end of TACSTD1 gene).

1.4.2 Familial CRC type X syndrome

According to large studies on HNPCC, mutations in genes of MMR were present only in about the half of the families met the Amsterdam criteria. In mutation negative cases the risk of CRC is only about twofold in contrast in families with germ cell mutations and the average age was higher at diagnosis of the tumor (61 vs. 49 years). Furthermore, the elevated risk was verified only for CRC while it was not significant in other tumors. Therefore it was suggested to use the term "Lynch syndrome" for families bearing mutations and the term "familial CRC type X syndrome" for families without genetic alterations.

1.5 Pathogenesis of CRC

The basis of the hereditary and also sporadic CRC is the genetic instability. It has basically three main mechanisms. Chromosome instability is the cause of the 85% of the sporadic cases and it is accompanied with several numeral and structural chromosomal malformations altering the functions of several important genes. The most frequently altered genes on this classic adenoma-carcinoma pathway are APC, K-ras, DCC, p53 and Src. The lack of MMR proteins (MLH1, MSH2, MSH6, PMS2) is responsible for Lynch syndrome representing about 5% of all CRC cases and for 8-15% of sporadic cases. Finally, in 10-20% of CRC a hypermethylation can be verified affecting several genes' promoter regions resulting in lack or decreased production of these proteins (e.g. APC, MLH1). This "CpG island methylator phenotype" can also be accompanied with microsatellite instability and can be detected mainly in sporadic cases.

1.6 Other potentially significant factors in the pathogenesis of CRC

The peroxisome proliferator-activated receptor genes (PPAR) can also take part in carcinogenesis. PPAR genes encode nuclear receptors regulating proteins involved in cell proliferation and lipid metabolism. Several data support the protective effect of cyclooxygenase inhibitors in development of CRC. Although the mechanism is not clear the role of COX-2 is supposed which is overproduced in CRC cells.

The possible role of Ki-67 protein in carcinogenesis is less known. It is widely used as a proliferation marker in histopathology. Presumably it can contribute to development of CRC by means of genetic alterations. The V617F mutation of JAK2 gene results in constant JAK2 activity, permanent intracellular phosphorylation and cell proliferation. The effects of the FLT3 gene's internal tandem duplication and point mutations in the tyrosine kinase domain are similar. Both genetic alterations play a role essentially in the development of hematological malignancies. They were not detectable in sporadic CRC and tumor cell lines but they have not been investigated particularly in familial/hereditary CRC.

1.6.1 Ki-67 protein

Antibodies against Ki-67 protein have been used to detect the ratio of the proliferating cells (Ki-67 index) for decades. They bind to a human nuclear antigen (Ki-67 protein) which is present in all proliferating cells (in phase G1, S, G2, M) but is absent in resting cells (in phase G0). The Ki-67 protein's biological functions are rather complex. It is able to bind to DNA and non-histon proteins and associates with DNA helicases, therefore it may have a role in regulation of developing and degradation of higher order structure of chromatin. It can influence the cell proliferation at several points. It promotes cells to enter phase S and the related DNA replication, the G2-M transition and induces

mitosis. It may have a role in repairing of the alterations of double stranded DNA. Finally, its interaction with ribosomal proteins plays a role in quick restoration of ribosomal functions after cell division. Based on these, changes in the structure of the protein may influence cell division and may play a role in carcinogenesis due to an uncontrolled, permanent cell proliferation.

1.6.2 V617F mutation of the JAK2 gene

JAK2 is a cytoplasmic protein tyrosine kinase which plays an important role in signalization of certain cytokines. After binding to the cytokine receptors it is autophosphorylated, becomes activated and phosphorylates downstream intracellular signaling elements. Regulation of cell division and cell survival gives importance to the JAK/STAT signaling pathway.

In exon 14 of the JAK2 gene, in the region of JH2 pseudokinase inhibiting JH1 domain the presence of the G1849T (V617F) mutation leads to permanent activation of the JH1 domain. This constant JAK2 activity results permanent intracellular phosphorylation and cell proliferation, playing role in development of hematological malignancies. This genetic alteration occurs most frequently in polycythemia vera (95%), essential thrombocythemia (50%) and idiopathic myelofibrosis (50%). Its presence was not proved in sporadic colon cancer and in colon tumor cell lines.

1.6.3 Internal tandem duplication (ITD) and mutation of tyrosine kinase domain (TKD) of FLT3

Fms-like tyrosine kinase 3 (FLT3) is a receptor tyrosine kinase. Its ligand is a transmembrane protein expressed on the surface of several cells and tissues. Ligand binding leads to dimer formation of the receptor and its autophosphorylation which induces cell proliferation through activation of intracellular signaling pathways and results inhibition of apoptosis.

Internal tandem duplications (ITD) in the juxtamembrane domain (exons 14 and 15) is the most frequent mutation of FLT3. The length of ITD varies between 3 and 400 base pairs, while the number of extra incorporated nucleotides varies between 3 and 36 base pairs. As reading remains in frame, the produced protein preserves its original kinase function, but – depending on the size of the tandem duplication – it has a longer JM domain. The other group of mutations involves the activation loop of the tyrosine-kinase domain (TKD). In 85-90% of these cases there is a mutation in codon 835, which results in an amino acid change. These mutations lead to spontaneous and permanent activation of the tyrosine-kinase, and finally result in cell proliferation and prolonged cell survival. Prevalence of genetic changes of FLT3, which is relatively high in AML, has not yet been examined in CRC.

2. AIMS OF THE STUDY

Our main goal is to identify new genetic variations in patients suffering from inherited and sporadic CRC. We focus our work on proteins, which either play a key role in the cell cycle or – due to mutations – induce cell proliferation. That is the reason why we chose Ki-67, JAK2 and FLT3 genes. While Ki-67 has an important role in cell proliferation even in normal cells, genetic variants of JAK2 and FLT3 usually occur in different myeloid malignancies. Except for JAK2, no mutation of these genes has ever been tested in sporadic CRC. None of these genes has been studied in inherited type of colon cancer.

We try to answer the following questions:

- Can we detect mutations of Ki-67 in different malignant cell lines and in DNA samples isolated from different colon cancers?
- We also aim to compare analytic parameters of the three widely used methods for the detection of FLTD3-ITD mutations (PCR + agarose or polyacrylamide gel electrophoresis or capillary electrophoresis). We aim to compare their applicability in routine laboratory diagnostics as well.
- Are JAK2 V617F, FLT3-ITD and – TDK mutations detectable in isolated DNA derived from colon cancers?

3. PATIENTS, MATERIALS AND METHODS

3.1 Characteristics of participating patients

Samples from surgical preparations of 26 patients with sporadic CRC (14 male, 12 female patients; age: 65.4±12.5 yrs) were used for testing Ki-67, FLT3 and JAK2 mutations. FLT3 and JAK2 mutations were also tested in 38 patients positive for Amsterdam- or modified Bethesda criteria. In these patients we performed MSI test and IHC examination of 3 MMR proteins (MLH1, MSH2, MSH6). In case of high MSI level and/or deletion of a MMR protein, the sequence analysis of MLH1 and MSH2 genes was performed. We found genetic aberrations in 6 patients (Lynch syndrome; 4 male, 2 female patients; age: 47.3±10.7 yrs); these cases were presented before. The remaining 32 patients (15 male, 17 female patients; age: 56.3±13.4 yrs) were classified as familiar CRC type X. All patients with HNPCC and sporadic CRC were followed by Miklós Tanyi, M.D., PhD. at the Department of Surgery, University of Debrecen MHSC. The preparation of samples and the IHC examinations were performed by László Tóth, M.D. at the Department of Pathology, University of Debrecen MHSC. The MSI test was performed at the Department of Laboratory Medicine, University of Debrecen MHSC. Mutation analysis of MLH1/MSH2 genes was performed by Judit Olasz, Ph.D. at National Institute of Oncology. Samples from 107 adult patients with acute myeloid leukemia (AML; 47 male, 60 female patients; age: 50.8±17.1 yrs) and 99 polycythemia vera (PV; 55 male, 44 female patients; age: 61.4±14.1 yrs) were used as controls for the examination of FLT3 and JAK2 mutations. These patients were followed by Péter Batár, M.D. and Prof. Miklós Udvardy M.D., Ph.D., DSc at the 2nd Department of Internal Medicine, University of Debrecen MHSC; and László Szerafin M.D., Ph.D. at the Department of Hematology, Jósa András Hospital, Nyíregyháza.

3.2 Cell lines

We used four cell lines for testing Ki-67 mutation: a cervix carcinoma (HeLa), a pulmonary carcinoma (A549) and two colon carcinoma (CXF94, SW480) cell lines (American Type Culture Collection). Cells were cultured in DMEM+10% FBS (Gibco-BRL) under standard conditions.

3.3 RNA, DNA isolation

We used 5 x 10^6 carcinoma cells for RNA isolation with TRIzol™ (Gibco BRL). From patients with sporadic and hereditary CRC we used formalin fixed, paraffin-embedded slides for DNA isolation. The 1-2 μm thick slides were deparaffinated by heating and with xylol, and then were rehydrated by a downward dilution of alcohol solutions. Preparations were digested with 20 μl Proteinase K (56ºC, 16-18 hrs), then we used the Tissue protocol of the "QIAmp DNA Blood mini kit" (Qiagen). For testing mutations of FLT3 gene we used blood from patients with AML, for testing mutations of JAK2 we used the granulocyte layer after Ficoll separation of blood from PV patients; DNA was isolated with „QIAmp DNA Blood mini kit" (Qiagen). Purity and quantity of DNA and RNA were checked by spectophotometry (GeneQuant or NanoDrop).

3.4 Sequencing of Ki-67 cDNA with cloning

RNA from cell lines (5 μg) was reverse transcribed to cDNA by using Murine Reverse Transcriptase enzyme (Ready-To-Go®, Amersham-Pharmacia) and 100 pmol $pd(T)_{12-18}$ (Amersham-Pharmacia). Coding sequences of Ki-67 gene were amplified by 4 PCRs. The sequence of primers, the composition of PCR mixes and the conditions of PCRs are discussed in the dissertation. PCR products were separated by electrophoresis on 1% agarose gel, and purified after cutting from the gel with Prep-A-Bene® DNA Purification System (BioRad Laboratories), or we used E.Z.N.A.® Cycle Pure Kit (PEQLAB Biotechnologie Gmbh) on samples from PCR tubes. Purified PCR products were inserted to a

pCR®II-Blunt-TOPO® vector (Invitrogen Corp.), then amplified in TOP 10 One Shot® competent *E. Coli* bacteria. Clones were selected on Kanamycin-containing medium, then isolated with „QIAGEN Plasmid Maxi Kit". We tested three clones from each cell line at least twice. For sequencing plasmid DNA we used the Thermosequenase Kit (Amersham Pharmacia) and M13 primers on a Model 4200 analyzer (LI-COR Inc.). We compared the sequence with the original article describing the cDNA of Ki-67 and the reference sequence from gene bank Ensembl.

3.5 Examination of the mutation Ki-67 c.1300delC in primary CRC samples by sequencing

The appropriate part of exon 7 of the Ki-67 gene was amplified by PCR. We used Microcon ultrafiltration column (Amicon® Ultra – 0,5 ml, 30K; Millipore) for the purification of the PCR product (437 bp). BigDye 3.1 sequencing kit (Applied Biosystems) was applied for the sequencing PCR and Qiagen DyEx Spin Kit for purification. The purified product was dryed, then stored in 15 µl formamide, at -20°C. We denaurated the samples immediately before sequencing. Sequencing was performed by ABI Prism 310 Genetic Analizer.

3.6 Detecting the FLT3-TKD mutation using PCR+RFLP

The appropriate part of exon 20 of the FLT3 gene was amplified by PCR. During digestion of the wild type allele the 114 bp PCR product splits to a 68 and a 46 bp product. In the presence of the mutation the recognition site of the restriction enzyme is altered and in this way a 114 bp PCR product can also be detected.

3.7 Detecting the FLT3 internal tandem duplication (ITD) using PCR and agarose or polyacrylamide gel electrophoresis

The appropriate part of exon 14-15 of the FLT3 gene was amplified by PCR. We visualized the PCR product using agarose (3%) or polyacrylamide (6%) gel electrophoresis. Wild type allele gives a 331 bp band; mutant allele gives another band with a higher size.

3.8 Detecting the FLT3 internal tandem duplication (ITD) using fragment analysis and sequencing

The appropriate part of exon 14-15 of the FLT3 gene was amplified by PCR, using fluorescent labeled primers. PCR product was denatured and stored in formamide at -20°C. Denatured mixes were analysed using an ABI310 capillary electrophoresis system. We used GeneScan for collecting data, GeneScan and PeakScanner software (Applied Biosystems) for analysis. Wild type allele gives a 331 bp peak, mutant allele gives a peak with a higher size. In 10 of the FLT3-ITD positive samples sequencing was also performed.

3.9 Detecting V617F mutation of JAK2 gene using PCR+RFLP

The appropriate part of the exon 14 of the JAK2 gene was amplified by PCR. Undigested and digested PCR products were visualized using agarose gel electrophoresis. Wild type allele gives a 174 bp and a 160 bp product, mutant allele gives a 364 bp product.

3.10 Microsatellite instability testing

During MSI testing we examined the stability of 3 dinucleotide and 2 mononucleotide microsatellites using PCR and CE. DNA fragments around the 5 microsattelites were amplified by 5 PCRs. Pooled PCR mixes and ROX-labeled size standard (ABI ROX 500, Applied Biosystems) was mixed in formamide and denaturated. Then we analyzed the samples with an ABI310

capillary electrophoresis system. If 1 of 5 microsatellites is instable, MSI is low, if at least 2 of 5 are instable, MSI is high.

3.11 Statistical analysis

We examined the normality of distribution of the studied parameters with Kolmogorov-Smirnov test. We performed the comparison of categorical variables with χ^2-test while that of continuous variables with Mann-Whitney U-test or Student t-test. Correlation of continuous variables was evaluated by Pearson test while patient survival was measured by Kaplan-Meier analysis. Any difference was significant if $p<0.05$. Statistical analysis was performed by software “Statistica”.

4. RESULTS

4.1 Revealing Ki-67 mutations in tumor cell lines and samples of patients with CRC

We isolated RNA from cell lines of two colon tumors (CXF94, SW480), a cervix carcinoma (HeLa) and a lung tumor (A549). We sequenced the Ki-67 cDNA with the help of 4 PCRs. During our investigations we have found eight genetic alterations. The first was the c.237>A nucleotide exchange in the CXF94 cells, which does not cause changes in the order of the amino acids. We have detected an A>G and a T>A exchange at positions 311 and 712, which resulted in an aspargine/serine and a tryptophan/arginine alteration in the CXF94/SW480 and the HeLa/CXF94 cell lines. In the position 1300 a one base pair deletion could be shown in the cells A549 and SW480. This change causes a frame shift mutation and a STOP codon in the twelfth codon from the deletion, which in turn results in the absence of protein or lead to a truncated protein. The exchanges of isoleucine/leucine (c.1891A>C) and that of arginine/tryptophan (c.2494C>T) were shown in the cell lines CXF94 and A549. Finally we could detect the exchange of c.9289A>G and that of c.9737G>C in all of the cell lines. Since all of the variants found except for c.1300delC proved to be a polymorphism, we examined only this genetic alteration in DNA samples isolated from the tumors of 26 patients with sporadic CRC. The c.1300delC deletion could not be detected in the examined patients with CRC.

4.2 Comparison of methods used for detection of FLT3-ITD

4.2.1 The prevalence of methods applied for detection of FLT3-ITD in the international literature

In the first step we aimed to find out, which methods can be applied for detection of FLT3-ITD and how often these methods are eventually applied in the international literature. In order to achieve our goal we performed a search in the PubMed data base with the combination of the following terms: “FLT3”

AND [“ITD” OR “LM” OR “internal tandem duplication” OR “length mutation”] AND “Language: English” AND “Publication year: 2010-2011”. A sum of 177 publications met these criteria. First we excluded 15 reviews and 27 papers dealing with basic research. We have managed to obtain the full text of 122 of the remaining 135 papers. Out of these, we could identify the method used for detection of FLT3-ITD in 99 cases.

Regarding the distribution of the methods, agarose (AGE) and/or polyacrylamide (PAGE) gelelectrophoresis have/has been used in 29 papers (29.3%), and in 37 cases (37.4%) at least one of these in combination with DNA-sequencing. The ratio of application of capillary electrophoresis (CE) in itself or in combination with DNA-sequencing was 16 and 3 (16.2% and 3.0%). The 14 remaining publications (14.1%) applied different combinations of these and other less frequently used methods. Based on our findings AGE and PAGE still seem to be widely applied methods for detection of FLT3-ITD, as a result their comparison with the capillary electrophoresis, and the characterization of these 3 analytic methods seemed to be interesting.

4.2.2 Detection of FLT3-ITD in the samples of patients with AML using three different electrophoretic methods

We have examined the detection of FLT3-ITD in 95 DNA samples of 73 adult patients with AML using three different electrophoretic methods. In the case of 18 patients we analyzed 2, and in the case of 2 patients 3 independent samples. We were able to demonstrate the presence of at least one mutant allele in 20 samples of 18 patients, independently from the method applied.

The ITD size defined based on the CE was between 9-178 base pairs (median 40 bp), the ratio of mutant/wild type alleles was between 0.006 and 3.714 (median 0,144), while the ration of mutant/all alleles was between 0.48 and 77.8% (median 12.5%). Out of the 18 ITD-positive patients in 9 cases more than one mutant allele could be shown (multiple ITD). Although all three

methods were capable of identifying the FLT3-ITD-positive samples, there were differences between them regarding the number, size and intensity of mutant alleles; accordingly in our further research we examined the analytical characteristics of the three aforementioned electrophoretic methods.

4.2.3 Defining the analytic parameters of the electrophoretic systems: reproducibility

For our research we used two ITD-positive samples (B35 and B80). During the CE the sample B35 showed a high and a hardly visible, lower mutant peak at the bps 387 and 339. In the case of sample B80 we observed a high mutant peak at bp 355 and another one with very low intensity at bp 398.

Regarding the size of the PCR products amplified from the wild type and mutant alleles, we observed an excellent intra- and inter-assay reproducibility (CV% was under 0.2%). The size of the ITD was given by the difference between the size of the mutant and the wild type allele. The intra- and inter-assay CV% were under 1 and 2 percents, when the size of the ITD was larger (over 24 bps; sample B35 – ITD2, sample B80 – ITD1 and ITD2). In the case of sample B35 ITD1 we observed a smaller 9 bps insertion with relatively higher intra- and inter-assay CV% (7 and 11%).

By defining the ratio of the mutant/wild type and mutant/all alleles we observed altering reproducibility results. In the case of sample B35 ITD2 and the sample B80 ITD1 the ratios of the mutant/all alleles were 65% and 12%, and the intra- and interassay CV%s were below 5%. The reproducibility was worse, when the mutant/all allele's ratio was between 1.2-1.5% (sample B80 ITD2), the intra-assay CV% was 10-14%, while the inter-assay CV% was 25%. When the mutant/all alleles ratio was below 1% (sample B35 ITD1), the reproducibility was not acceptable any more; the CV% was over 50%.

The same examinations were also conducted in the case of AGE and PAGE. The PCR-product amplified from the larger mutant allele was easily

detectable at approximately the same place with the similar intensity, while the smaller mutant band was missing.

4.2.4 Defining the analytic parameters of the electrophoretic systems: sensitivity

In a further step we compared the detection limits of the three different electrophoretic methods. We made a serial dilution from the DNA of sample B35 and B80 with the DNA of an ITD-negative sample. We defined the mutant/ all alleles ratio in the original sample with the help of CE and PeakScanner analysis, and in the diluted DNA samples we calculated it on the basis of the starting value and the dilution factor. We amplified the DNA mix with unmarked primers during the FLT3-PCR, and then we loaded 10 µl PCR products on agarose or polyacrylamide gel. The dominant mutant allele was detectable at 1-2% dilution with the help of both methods, but the minor mutant allele was not detectable by using these methods.

The diluted DNAs were also amplified with fluorescently labeled forward and unmarked reverse primer, which was followed by the denaturation of the PCR product, and later on by CE. We determined the mutant/wild type and the mutant/all allele's ratio by PeakScanner analysis. In order to define the lowest point terminating the linear part of the dilution curve we tested a variable cutoff value (0.1-0.5%) in the case of the calculated mutant/all allele's ratio and another value (0.001-0.005) in the case of the calculated mutant/wild type allele's ratio. We drew a linear regression line across the points above the respective cutoff. In the case of the two examined ratios the best regression coefficients (R=0.989 and R=0.990) belonged to the 0.2% and 0.002 cutoff. We defined the analytical sensitivity of the assay taking into account the ten data points having the lowest calculated mutant allele ratio. The mean+3SD of these values were 0.28% and 0.0028, which defines the detection limit of the measured mutant/all and mutant/wild type alleles ratio.

4.2.5 Defining the analytic parameters of the electrophoretic systems: resolution

In the sample B114 the 9 bps difference between PCR products of the wild type and the mutant alleles was detected by each method. On the other hand, in the case of sample B147 the 370 and 373 bp mutant alleles were equally well detectable with EC, but this three bps difference was not resoluble with AGE or PAGE.

4.2.6 The analysis of multiplex bands detected with the help of AGE and PAGE in FLT3-ITD-positive samples

In some of the 20 DNA samples, which had been found to be FLT3-ITD-positive, we demonstrated 2, 3 or even 4 large-sized mutant bands with the help of PAGE and AGE, most of which were not detectable in the case of CE. We examined these in more detail in the sample of patient B55. We cut the wild type (band 1= 331 bp), the mutant 1 (band 2 = approx. 350 bp) and the mutant 2 (band 3 = approx. 550 bp) out of polyacrylamide gel, then we defined the base sequence of these with the help of bidirectional sequencing. Based on these, a 21-bp-duplication has been proven in the region of the 14. intron/15. exon (c.1773_1793 dup21) in the band of mutant 1. In the band of mutant 2 the mixture of the two sequences was detectable even after the multiple excision and amplification of the band. With the help of the joint amplification of the DNA isolated from these two bands and of denaturation/renaturation experiments we have managed to prove that the band of mutant 2 was the heteroduplex of the bands of wild type and mutant 1.

4.2.7 Comparison of ITD sizes measured with CE and DNA-sequencing

It is well known from earlier publications, that ITD always causes „inframe” changes in the FLT3 gene. This phenomenon also means that the

number of nucleotides in ITD can always be divided by three. Surprisingly this statement does not always stand for ITD size measured with CE by us. Therefore, in 10 positive cases we also directly sequenced exon 14-15 of the FLT3 gene. Correlation between the two methods was strong (R=0,999), but the size of the insertion measured with DNA sequencing, turned out to be 7% larger compared to CE.

4.3 Prevalence of FLT3-ITD and -TKD mutations in tumor samples removed from patients suffering from sporadic and inherited forms of colon cancer

Presence of FLT3 mutations was examined in tumor samples removed from patients, of whom 26 were suffering from sporadic and 38 from inherited CRC.

Out of the 38 inherited cases 6 patients were suffering from Lynch-syndrome, and the rest from familiar CRC X type. When we compared clinical characteristics of inherited and sporadic types of CRC, average age turned out to be lower and mean survival significantly higher in patients suffering from Lynch syndrome compared to sporadic type CRC. We found a significantly lower average age, longer mean survival and more frequent left colon involvement in the familiar CRC X syndrome group compared to patients in the sporadic group.

Concerning FLT3-ITD and TKD mutations, we could prove their presence nor in the sporadic, neither in the inherited group. In our control group of 107 patients, suffering from AML, presence of FLT3-ITD was 25.2% (27/107), while FLT3-TKD was present in 5.6% (6/107) of the cases.

4.4 Prevalence of JAK2 V617F mutation in tumor samples removed from patients suffering from sporadic and inherited types of colon cancer

We also examined the presence of V617F mutation of JAK2 with PCR+RFLP in the two groups. V617F mutation of JAK2 could be detected neither in the inherited, nor in the sporadic group. Our control samples were obtained from 99 patients suffering from PV. Frequency of V617F mutation was 86.9% (86/99).

5. DISCUSSION

5.1 Potential role of pKi-67 in development of CRC

Our results clearly verify that several genetic alterations of Ki-67 gene can be identified in different tumor cells' cDNA. Effect of c.1300delC seems to be most apparent as it results in a STOP signal in the codon 446 leading to a truncated protein. The other observed alterations are genetic polymorphisms associated with amino acid change occurring with relatively high frequency in the normal population. Merely on the basis of chemical characteristics of the exchanged amino acids the isoleucine/leucine and aspartate/asparagine exchange seem to be the least while tryptophan/arginine exchange the most emphasized because of differences between size and charge of their side-chains. Further two alterations may affect potential phosphorylation sites. Based on these, verified polymorphisms may influence function of the protein but studies conducted on larger population with CRC are needed to decide if they have a role in carcinogenesis. As the c.1300delC may have the most unequivocal effect we studied its presence in samples of patients with sporadic and hereditary CRC but this alteration could not be identified.

Interestingly, as many publications apply Ki-67 expression to determine the proportion of proliferating cells as few studies deal with biochemical and functional characteristics of the pKi-67. Besides our data genetic alteration in the gene of Ki-67 has not been published. Presumably genetic alterations causing complete absence of the protein or its function can cause death of the given cell. Presence of c.1300delC resulting in a STOP codon published by us does not contradict this because this is in the exon 7 and there is a more frequent splice variant of the pKi-67 not containing this exon. Presumably changing of the rate of Ki-67 isoforms may have an impact on cell proliferation and may have a role in malignant transformation.

5.2 Evaluation of analytical parameters of electrophoretic methods used for detection of ITD in FLT3 gene

FLT3-ITD is one of the most frequent genetic alterations in adult patients with AML. The most frequently used three methods to detect ITD in routine diagnostics are PCR followed by agarose, polyacrylamide or capillary electrophoresis. In our study we tested these three methods with DNA samples of patients with AML and evaluated their analytical parameters.

Rate of the ITD-positive samples was the same with all three methods referring to similar sensitivity of them. At the same time CE also verified some less intensive mutant bands not visible with AGE and PAGE. According to this the detection limits of the methods were 1-2% in case of AGE and PAGE and 0.28% in case of CE. Majority of the former publications with FLT3-ITD established similar sensitivity in case of AGE and PAGE but two articles published slightly higher limit (5-6%). Regarding to CE 0.8-2% sensitivity was published in five papers and 3-10% in other four articles. The slightly higher sensitivity of CE observed by us can be explained by the lower RFU limit that we used for defining the peaks. In the case of new samples we accepted RFU>50 as a peak and during follow-up samples with known peak position even RFU>15 was acceptable. By contrast former publications used RFU>150 as a limit. We repeated our examinations in order to demonstrate the presence of these ITDs with low signals and we only accepted them as positive if the presence of the peaks was reproducible. Despite the relatively low detection limit only mutant allele rate above 1.5% could be measured with acceptable reproducibility therefore we accepted this value as the quantitation limit.

Although these mutant alleles with very low rate were not detectable with AGE and PAGE we have found several extra bands with these methods which were absent in CE electrophoretograms. These extra bands with larger size studied by DNA sequencing and heteroduplex analysis corresponded to

heteroduplexes of the wild-type and mutant alleles. Former papers also published this phenomenon.

We compared the resolution of the different electrophoretic methods, too. Although all of them are able to detect a difference of 9 bp between bands/peaks only CE can resolve a 3 bp difference. Two former publications also observed similarly high resolution supporting the importance of this technique in detection of multiple ITDs.

Rate of multiple ITDs in our study determined by CE was 50% (9/18) that is slightly higher than the previously published 11-32%. It can also be explained by the lower RFU threshold used by us for definition of peaks during CE.

Regarding to ITD's size basically three different methods were used to determine it. ITD sizes of 3-400 bp were found by DNA sequencing, CE and Agilent Bioanalyzer with an average of 24-70 bp. Our results are also between these borders (9-178 bp with average of 40 bp). We found that reproducibility of ITD size determination by CE is appropriate because even in the case of the shortest fragment (9 bp) we calculated 11% CV%. However, the number of the duplicated/inserted nucleotides was not divisible with three in every case which was already published in two former publications. This phenomenon can be explained with variability of size of PCR products amplified from the wild-type and mutant alleles causing ±1.0 bp change in ITD size. Because of this either the direct sequencing of the DNA sample or the sequence analyses of the isolated or cloned ITD fragments can provide exact size determination. Using the former method we sequenced ten samples containing ITDs, and ITDs that we found never caused a frame shift. At the same time the sizes measured by sequencing were slightly bigger than those were with CE. On the basis of literature data and our experience we suggest the use of DNA sequencing for exact ITD size determination particularly in view of new data about the prognostic importance of location of the duplication/insertion.

More than 20 papers studied the rate of mutant alleles in samples of patients with AML using four different methods (semiquantitative AGE or PAGE in 5 papers, dHPLC, Agilent Bioanalyzer and CE in 1, 2 and 13 papers). Mean value of the ratio of mutant/wild-type alleles was 0.144 in our patient group while that of mutant/total alleles was 12.5%. It is slightly lower than previously published data (mutant/wild-type allele ratio: 0.530-0.836, mutant/total allele ratio: 30-47%). Presumably this can also be explained by the lower RFU threshold used for determination of peaks. Regarding to reproducibility of these measurements our data shows that mutant/wild-type and mutant/total allele ratio above 0.020 and 1.5% can be characterized by <25% CV%, therefore, we accept these values as quantitation thresholds of the method.

5.3 Significance of the JAK2 V617F, FLT3-ITD and -TKD mutations in sporadic and hereditary CRC

Although JAK2 gene mutations associated mainly with chronic myeloproliferative disorders including first of all polycythemia vera several experimental data suggest the possible role of activated JAK/STAT signaling in the pathogenesis of CRC. Activated, phosphorylated variant of STAT3 can be detected in an increased amount in the nucleus of tumor cells associated with more pronounced invasiveness and progression of the tumor and worse prognosis. Inhibitors of JAK2/STAT3 decreased the proliferation and invasiveness of the tumor cells.

On the basis of this we can presume that mutations of JAK2 and related genes causing constant signaling and activity may play a role in development of CRC. In two large studies on the proteins of the JAK/STAT system the JAK1 p.E886K and TYK2 p.H732R mutations were detectable in CRC with low incidence. Four studies examined directly the presence of JAK2 V617F mutation in CRC tumor cell lines and in samples of patients with sporadic CRC

but the mutation could not be detected. Our results also verify that this alteration do not occur in sporadic CRC.

The JAK2 V617F mutation has not been tested in hereditary CRC. We studied samples of 6 patients with Lynch syndrome and 32 ones with familiar CRC type X but JAK2 V617F mutation was not present.

Lack of JAK2 V617F mutation can be explained in many ways. Maybe the sensitivity of the method of PCR + RFLP used for detection of this alteration is inappropriate and not able to detect mutant alleles present in low amount. As the DNA isolation was performed dominantly from tumor tissue of the paraffin sections this is not probable. Other possibility is that further genetic alterations may be present in JAK2 gene e.g. mutations in exon 12 and other rare alterations not studied by us that can induce the JAK/STAT signaling pathway. Furthermore epigenetic alterations of JAK2 and other signaling elements may influence their behavior. Finally, lack of certain negative regulating elements (SOCS proteins) can result increased JAK/STAT activation.

Expression and mutations of FLT3 occur dominantly in hemopoetic cells but it can also be detected in a relatively high amount in placenta and in the central nervous system. Expression of FLT3 is much less in other tissues e.g. in colon. As previously similar examination has not been performed we determined the prevalence of FLT3 mutations in samples of patients with sporadic and hereditary CRC but genetic alterations were not detectable. The sensitivity of used methods seems to be appropriate as frequency of FLT3-ITD and –TKD alterations in the control AML population was 25.2% and 5.6% in accordance with the literature data.

Mutation analysis of the 6 patients with Lynch syndrome was also interesting from other aspects. Mutations of FLT3 and JAK2 genes were also not detectable in these patients so their formation does not seem to be probable even in the case of loss of function of the MMR genes and they probably do not play a role in the pathogenesis of neither sporadic nor hereditary CRC.

6. SUMMARY

Colorectal carcinoma is the second most frequent cancer cause of death in the majority of the developed countries. Environmental and genetic factors – many of which have been successfully identified – may play a role in the development of sporadic and hereditary CRC, too. In our study we analyzed samples of patients with sporadic and hereditary CRC searching for genetic alterations of Ki-67, FLT3 and JAK2 genes. We focused on such proteins which either play an essential role in regulation of cell cycle or in the case of their mutations induce an enhanced cell proliferation. Genetic alterations we studied have not been examined by others in patients with hereditary CRC and there are only limited data according to sporadic cases, too.

Ki-67 protein has a role in the regulation of higher chromatin structure and cell cycle, therefore lack of the protein or alteration of its structure may play a role in the development of certain tumors. Analyzing four tumor cell lines we have successfully identified 8 genetic alterations in the Ki-67 gene one of which – a one-nucleotide deletion that was not described earlier – was resulted in a frame shift and truncated protein, one mutation was not associated with amino acid change while other 6 alterations were polymorphisms with amino acid changes. We also searched for the presence of c.1300delC mutation in samples of 26 patients with sporadic CRC but it was not detectable.

FLT3-ITD, -TKD and JAK2 V617F mutations are frequent genetic alterations accompanying to myeloid malignancies. As a first step we validated three genetic methods used for detection of FLT3-ITD which had not been published in such detailed way previously. Our results suggest that CE is the most suitable method to detect and quantify the mutant/total allele ratio of FLT3-ITD. Verification of the presence of multiple ITDs is possible with CE if the ratio of the mutant alleles is above 0.28% while DNA sequencing can provide reliable information about the exact size and place of ITD. The frequency of FLT3-ITD and –TKD mutation was 25.2% and 5.6% in the control

group of 107 AML patients while the rate of JAK2 V617F mutation was 86.9% in 99 PV patients similarly to data published in the literature. Thereafter we analyzed the presence of FLT3-ITD and mutations of FLT3-TKD and JAK2 V617F in samples of 26 and 38 patients with sporadic and hereditary CRC but we could not have verified these alterations. Mutations of FLT3 and JAK2 genes were also not detectable in 6 patients with Lynch syndrome so their formation does not seem to be probable even in the case of loss of function of the MMR genes and they probably do not play role in the pathogenesis of neither sporadic nor hereditary CRC.

7. NEW SCIENTIFIC RESULTS OF THE DISSERTATION

7.1 We have found 8 genetic alterations in cDNA of tumor cell lines 7 of which proved to be polymorphism occurring more or less frequently in the normal population. However, a one nucleotide deletion (c.1300delC) may have theoretically direct pathogenetic effect through creating STOP codon which results production of a truncated protein.

7.2 Presence of c.1300delC could not be verified in samples of patients with sporadic CRC.

7.3 We validated 3 genetic methods used for detection of FLT3-ITD which had not been published in such detailed way previously. Our results suggest that

- CE is the most suitable method to detect and quantify the mutant/total allele ratio of FLT3-ITD
- in the case of CE the analytical sensitivity of detection of mutant/total allele ratio can be increased up to 0.28%.
- verification of the presence of multiple ITDs is possible with CE
- DNA sequencing can provide reliable information about the exact size and location of ITD

7.4 FLT3-ITD and mutations of FLT3-TKD and JAK2 V617F did not occur in samples of patients with sporadic and hereditary CRC

8. LIST OF PUBLICATIONS

UNIVERSITY AND NATIONAL LIBRARY UNIVERSITY OF DEBRECEN
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Register Number: DEENKÉTK/297/2012.
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Subject: Ph.D. List of Publications

Candidate: Tamás Bubán
Neptun ID: UBS096
Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. **Bubán, T.**, Koczok, K., Földesi, R., Szabó, G., Sümegi, A., Tanyi, M., Szerafin, L., Udvardy, M., Kappelmayer, J., Antal-Szalmás, P.: Detection of internal tandem duplications in the FLT3 gene by different electrophoretic methods.
Clin. Chem. Lab. Med. 50 (2), 301-310, 2011.
DOI: http://dx.doi.org/10.1515/CCLM.2011.762
IF:2.15

2. **Bubán T.**, Tóth L., Tanyi M., Kappelmayer J., Antal-Szalmás P.: Ki-67: Egy régi játékos új arcai.
Orvosi Hetilap. 150 (23), 1059-1070, 2009.
DOI: http://dx.doi.org/10.1556/OH.2009.28638

3. **Bubán, T.**, Schmidt, M., Broll, R., Antal-Szalmás, P., Duchrow, M.: Detection of mutations in the cDNA of the proliferation marker Ki-67 protein in four tumor cell lines.
Cancer Genet. Cytogenet. 149 (1), 81-84, 2004.
DOI: http://dx.doi.org/10.1016/S0165-4608(03)00297-8
IF:1.577

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List of other publications

4. Tanyi, M., Olasz, J., Tanyi, J.L., Tóth, L., Antal-Szalmás, P., **Bubán, T.**, András, C., Urbancsek, H., Garami, Z., Csuka, O., Damjanovich, L.: Q48P mutation in the hMLH1 gene associated with Lynch syndrome in three Hungarian families.
Fam. Cancer. 11 (3), 519-524, 2012.
DOI: http://dx.doi.org/10.1007/s10689-012-9515-9
IF:1.302 (2011)

5. Tanyi, M., Olasz, J., Lukács, G., Tanyi, J.L., Tóth, L., Antal-Szalmás, P., Ress, Z., **Bubán, T.**, András, C., Damjanovich, L.: A new mutation in Muir-Torre syndrome associated with familiar transmission of different gastrointestinal adenocarcinomas.
Eur. J. Surg. Oncol. 35 (10), 1128-1130, 2009.
DOI: http://dx.doi.org/10.1016/j.ejso.2009.03.011
IF:2.564

6. Bodnár, Z., **Bubán, T.**, Várvölgyi, C.: Submucosal tumor-like gastric wall abscess.
Gastrointest. Endosc. 59 (4), 599-599, 2004.
DOI: http://dx.doi.org/10.1016/S0016-5107(03)02724-X

7. Várvölgyi, C., **Bubán, T.**, Szakáll, S., Hargitai, Z., Galuska, L., Jeney, C., Kakuk, G., Gaál, J.: Fever of unknown origin with seronegative spondyloarthropathy: An atypical manifestation of Whipple's disease.
Ann. Rheum. Dis. 61 (4), 377-378, 2002.
DOI: http://dx.doi.org/10.1136/ard.61.4.377
IF:3.593

8. Várvölgyi C., Mátyus J., Balogh Z., Bodnár Z., **Bubán T.**, Kakuk G.: Szűrővizsgálatok a gastroduodenalis fekélybetegség kockázatának felmérésére vesetranszplantációs listán lévő betegekben.
Magyar Belorv. Arch. 52 (4), 339-344, 1999.

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The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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