Ph. D. THESIS

Role of genetic alterations and promoter methylation of $p16^{INK4A}$ and $p14^{ARF}$ tumour-suppressor genes in CML acceleration and tumourigenesis of EBV-infected B-cells

Nagy Etelka

Tutor: Dr. Zoltán Beck

University of Debrecen
Medical and Health Science Center
Faculty of Medicine
Department of Medical Microbiology

2005

INTRODUCTION

The *INK4A* gene localized on chromosome 9p21 uniquely encodes two distinct tumour suppressors by using separate promoters and alternative reading frames. The two distinct proteins encoded by the *INK4A* gene are specified by translating the common second exon in alternative reading frames. The cyclin-dependent kinase inhibitor $p16^{INK4A}$ is specified by an RNA comprising exons 1α, 2 and 3. The alternative product, designated $p14^{ARF}$ for „alternative reading frame", is encoded by the slightly smaller β transcript that comprises exons 1β, and 2.

The $p16^{INK4A}$ protein induces a G_1 cell cycle arrest by binding to and inhibiting the activity of cyclin-D-dependent kinases CDK4 and CDK6, thereby maintaining the Rb protein in its growth suppressive, hypophosphorylated state. The $p14^{ARF}$ protein restrains cell growth by abrogating MDM2 inhibition of the p53 activity, resulting in a distinctive cell cycle arrest in both the G_1 and G_2/M phases.

The $p16^{INK4A}$ gene is inactivated by mutations, homozygous deletions, or gene methylation in many tumours of diverse origin. Mutations of *INK4A/ARF* locus affect $p16^{INK4A}$ exclusively or together with $p14^{ARF}$, whereas homozygous deletions at the exon 2 at the *INK4A/ARF* locus affect both $p16^{INK4A}$ and $p14^{ARF}$. The $p14^{ARF}$ expression is under the control of its own promoter that can be silenced by methylation of CpG islands.

The role of $p16^{INK4A}$ and $p14^{ARF}$ gene alterations in the progression of CML is largely unknown. No deletions of $p16^{INK4A}$ were found in chronic phase (CP) of CML, whereas homozygous deletions of $p16^{INK4A}$ exons were detected in lymphoid blast crisis (BC) but not in myeloid BC. Mutations in $p16^{INK4A}$ were reported in one out of four CML cases who had BC, but lineage type was not mentioned. We studied DNA alterations and methylation status of $p16^{INK4A}$ and $p14^{ARF}$ genes in 30 patients with Ph chromosome-positive CML. All these patients were studied both at diagnosis and at the onset of accelerated phase (AP) to determine the possible role of alterations of $p16^{INK4A}$ and $p14^{ARF}$ genes in the transition of CML from CP to AP.

The oncoproteins of DNA tumourviruses play an important role in the regulation of cell cycle. In the second study we examined different EBV-positive and negative tumourigenic cell lines with Burkitt lymphoma origin, LCLs , and normal B-lymphocytes. Although some discrepancies exist concerning the frequency with which these genes are deleted, mutated or promoters are methylated, loss of $p16^{INK4A}$/$p14^{ARF}$ function by either genetic or epigenetic way appears to be important in development of Burkitt lymphomas and,

perhaps, LCLs. We studied genetic and promoter methylation changes affecting the *INK4A/ARF* locus in order to determine the frequency of these alterations in both tumour-suppressor genes, and show whether or not these changes are characteristic to a definite stage of tumourigenesis.

AIMS

We wanted to know the relationship between genetic and epigenetic inactivation of INK4A tumour-suppressor gene and CML progression and tumourigenesis of B-cell. We addressed the following issues:

-Does the inactivation of $p16^{INK4A}/p14^{ARF}$ genes take part in the CML progression? What kinds of mechanisms are involved in the inactivation and how frequent?

-What kinds of genetic alteration play a role? Do the mutation affect both alleles? Has any mutational hot spot?

-How many samples of CML contain the methylation of both genes?

-What is the cytogenetic background in the CML?

-How the EBV influences the alterations of *INK4A* gene in tumour cell lines?

-Are there any differences the inactivation of $p16^{INK4A}/p14^{ARF}$ genes in EBV associated BL lines, de novo transformed LCLs, immortalized LCLs and normal B-cells?

-Does the genetic or epigenetic alterations play more important role in the tumourigenesis of B-cells?

-Which phase of lymphomagenesis result the inactivation of tumour-suppressor gene?

MATERIALS AND METHODS

Isolation of granulocytes

Peripheral blood samples were obtained from leukaemia patients and normal, healthy individuals. The phase of CML was determined by standard clinical and hematological criteria. The CP specimens were collected at presentation, prior to initiation of treatment. The AP specimens were from patients who had received treatment during the CP of their disease, but had received no treatment after the onset of the AP prior to collection of the samples used in this study. A simple one-step method for purification of human granulocytes, based on the use of a discontinuous gradient of Percoll, was used.. The purity of granulocytic elements was higher than 97%.

Cell lines

For our second experiments EBV-negative BL cell lines, BL 41, Ramos; EBV-positive BL cell lines, AG 876, Akata, Akuba, Daudi, Jijoye, Mutu III clone 99, Namalwa, Rael, Raji, earlier established LCLs, CB-M1-Ral-STO, Cherry, IARC 171, JY, LCL-721; and freshly established LCL_{Mutu}, LCL_{Akuba}, $LCL_{,Jijoye}$, LCL_{Rael} were used.

Isolation of primary B-cells

Human primary B-cells were isolated from peripheral blood. Buffy coat were subjected to centrifugation over Ficoll paque gradients then the CD-19 positive B-cells were separated with pan-B Dynabeads M450.

De novo immortalisation of primary B-cells

EBV strains were purified from each examined EBV-positive BL cell lines treated with tetra phorbol acetate (TPA). The virus was pelleted by ultracentrifugation. To establish LCLs the freshly isolated B-lymphocytes were infected with EBV strains isolated from different BL cell lines at a low multiplicity of infection.

Polymerase chain reaction (PCR)–single strand conformation polymorphism (SSCP) analysis

DNA of granulocytes, BL cell lines, LCLs and normal B-cells was isolated using a QIAGEN blood kit. The $p16^{INK4A}$ and $p14^{ARF}$ genes were analysed for deletions by PCR-SSCP analyses. After PCR was performed, products were visualized under UV illumination. For SSCP analysis, PCR products were heat-denaturated, treated with formamide and loaded into a neutral 7.5 % polyacrylamide gel. Finally, bands were visualized by silver nitrate staining.

Automatic sequencing of $p14^{ARF}$ exon 1β, $p16^{INK4A}$ exon 1α, 2 and 3

The sequences of exons were analysed in each CML sample, BL cell line and LCL. Exons were amplified, processed and sequenced using an ABI Prism Big Dye Terminator Ready Reaction Kit and an ABI Prism 310 Automatic Sequencer.

Methylation analysis of $p16^{INK4A}$ and $p14^{ARF}$ genes

Promoter hypermethylation of the $p16^{INK4A}$ and $p14^{ARF}$ genes was determined by methylation-specific PCR (MSP). First, genomic DNA is modified with sodium bisulphite. While this treatment changes the unmethylated but not the methylated cytosines into uracil residues, specific primers can distinguish these alterations and PCR amplify only the adequate sequences.

Cytogenetic study

The karyotype of leukaemia cells was investigated by using standard cytogenetic procedures. Chromosomes were identified using G and Q banding techniques.

RESULTS AND DISCUSSION

Haematologic and cytogenetic characteristics of patients

We examined 30 patients with CP or AP of CML. All patients had significant granulocytosis. At the onset of AP blast cells occasionally appeared in the blood. Other features associated with AP disease included the presence of a high percentage of basophils, thrombocytosis and anaemia.

Apart from the presence of the Ph chromosome, the karyotype was normal in all patients at the time of first examination. At the onset of the AP of the disease, further chromosomal abnormalities in addition to the Ph were demonstrated in 14 of 30 patients. Loss of chromosome 13 or loss of the long arm of chromosome 13 were found in 3 cases. Loss of chromosome 17, the presence of an isochromosome of the long arm of chromosome 17 or shortening of the short arm of chromosome 17 were observed in 5 cases. A second Ph was found in 2 cases. The other chromosomal aberrations included trisomy 8, 19, or 21, loss of chromosomes 5, 7, 18, 20, or 21, and markers 2q- and 16p-. In 11 patients more than one chromosomal abnormalities were found.

De novo established LCLs

We managed to establish 4 LCLs (LCL_{Mutu}, LCL_{Akuba}, $LCL_{,Jijoye}$, LCL_{Rael}) from normal resting B-cells by *in vitro* infection with EBV originated from Mutu, Akuba, Jijoye and Rael cell lines. The various LCLs containing different isolates of EBV were designated so as to indicate the BL cell line from which the EBV was derived.

Deletion analyses of $p16^{INK4A}$ and $p14^{ARF}$ genes

PCR-SSCP analysis using DNA from 30 patients with CP or AP of CML, 2 EBV-negative or 9 positive BL cell lines, 4 *de novo* established LCLs, 5 immortalised LCLs revealed that none of samples had homozygous or heterozygous deletions of the $p16^{INK4A}$ or $p14^{ARF}$ genes.

Mutations in $p16^{INK4A}$ and $p14^{ARF}$ genes

Although PCR-SSCP analysis is suitable for detecting single base mismatches in addition to deletion, sensitivity of this method is inversely proportional to the size of the amplified product. DNA sequencing analyses detected mutations in 17 AP samples but in none of the CP samples. Peaks in the sequence chromatograms showed that the mutation was heterozygous in each case. Since no mutations were found in CP samples prior to initiation of treatment, it cannot be excluded that these mutations were induced mostly by chemotherapy given in the CP of CML.

We found point mutations in exon 1 of $p16^{INK4A}$ in 10 patients. A G→A transition at codon 12 was present in one patient, but did not result in an amino acid change. As the nucleotide substitution was not seen in the CP of CML, it was considered to be a silent mutation rather than a polymorphism. A specimen had a point mutation (GCG→GTG) in codon 26, resulting an Ala to Val amino acid substitution. In 6 AP samples codon 35 was changed from AGT to ATT resulting in an amino acid change from Ser to Ile. The remaining two patients had a nonsense mutation of GAG to TAG at codon 18.

Mutations in exon 2 of $p16^{INK4A}$ were detected in 11 patients. In five samples a silent change from Glu (GAG) to Glu (GAA) was observed. Missense mutations in codons 61, 105, 123 or 130 were detected in six cases. Alterations of two bases at codons 61 (GAG→CAG) and 105 (CTG→ATG) resulted in amino changes from Glu to Gln and Leu to Met, respectively. In two cases codon 123 was changed from CGC to CAC resulting in a substitution of Arg with His. In two samples mutation was at codon 130 (AGA→ATA), resulting in a change of Arg to Ile.

Because $p16^{INK4A}$ and $p14^{ARF}$ share exon 2 with different open reading frames, a silent mutation of GAG to GAA at codon 80 of $p16^{INK4A}$ resulted in a missense mutation of GGG to AGG at codon 103 of $p14^{ARF}$. Missense mutations identified in codons 61 and 105 of $p16^{INK4A}$ resulted in missense mutations at codons 83 and 127 of $p14^{ARF}$.

Two mutational hot spots of the *INK4A/ARF* locus have been detected. One of them was found at codon 35 in exon 1 of $p16^{INK4A}$. The other mutation was located to codon 80 in exon 2 of $p16^{INK4A}$. These mutational hot spots with loss of heterozigosity may play important role in inactivation of tumour-suppressor proteins.

Mutations in exon 1 of $p14^{ARF}$ were found in 3 patients. In two cases a missense AGG to AAG mutation (Arg→Lys) was observed in codon 21. In a third AP sample codon 34 was changed from TGG to AGG resulting in a substitution of Trp with Arg.

No mutations were detected in exon 3 in any case.

We also determined nucleotide sequences of each exon of the INK4A/ARF locus in 9 EBV-positive and 2 EBV-negative BL lines by direct DNA sequencing. As a result, we found 1 heterozygous point mutation in exon 2 affecting both tumour-suppressor genes and 2 heterozygous mutations in exon 1 of $p16^{INK4A}$. In Namalwa a G→T transition was detected at codon 98 resulting an Val to Leu amino acid substitution. This mutation resulted in a different, but also missense mutation at codon 120 of $p14^{ARF}$. Mutation in exon 1 of $p16^{INK4A}$ was detected in Mutu at codon 27 (GGG→TGG) resulting an amino acid change from Gly to Trp. Daudi had a nonsense mutation of GAG to TAG at codon 18. No mutations were detected in exon 3 and $p14^{ARF}$ exon 1 in any BL cell lines. None of the freshly established LCLs or primary B-cells showed sequence alteration of examined tumour-suppressor genes.

Methylation status of the $p16^{INK4A}$ and $p14^{ARF}$ genes

The CP and AP samples, EBV-positive or negative BL cell lines, all LCLs and normal B-lymphocytes were analysed for $p16^{INK4A}$ and $p14^{ARF}$ promoter methylation by using MSP. There was no detectable methylation of the $p16^{INK4A}$ and $p14^{ARF}$ genes in any of the samples from the CP of CML. We found a similar frequency of promoter methylation in the AP of CML for both genes. Methylation of the $p16^{INK4A}$ promoter was detected in 12 of the 30 AP samples. Ten AP samples with $p16^{INK4A}$ methylation also had a methylated $p14^{ARF}$ gene. Methylation of the $p14^{ARF}$ promoter alone was found in 2 AP samples. Simultaneous appearance of point mutations and the methylated status was observed in 8 AP samples, whereas 6 AP samples showed aberrant methylation without sequence alterations.

The aberrant promoter methylation correlates with lack of $p16^{INK4A}$ and $p14^{ARF}$ protein expression and results in collapse of the Rb and p53 growth control pathways, thus, the tumour cells with inactivated $p16^{INK4A}$ and $p14^{ARF}$ genes may have a selective growth advantage. In conclusion, our results indicate that $p16^{INK4A}$ and $p14^{ARF}$ are primary targets for inactivation in acceleration of CML, and aberrant promoter methylation is the main event underlining $p16^{INK4A}$ and $p14^{ARF}$ inactivation

We found the $p16^{INK4A}$ promoter to be completely or partially methylated in all BL lines. These BL cells that carried $p16^{INK4A}$ inactivation included both EBV-positive and negative cultures. Partial methylation of the $p14^{ARF}$ promoter was detected only in Ramos, while the other BL cell lines were unmethylated for this region. Both promoters were methylated in 4 of the 5 LCLs having surpassed 160 PDLs, but only the $p16^{INK4A}$ promoter in

1 “old” LCL. The status of both genes was normal in all *de novo* established LCLs, and there was no detectable methylation of the $p16^{INK4A}$ and $p14^{ARF}$ genes in normal B-cells.

In summary, our results suggest that evolution of EBV-transformed LCLs is a multistep process involving the inactivation of tumour-suppressor genes, and result in loss of cellular growth control. The inactivation of $p16^{INK4A}$ tumour suppressor gene in BL cell lines and both $p16^{INK4A}$ and $p14^{ARF}$ genes in LCLs by epigenetic machinery may be an important hallmark of these tumours corresponding immortalisation.

SUMMARY

1. .The genetic and epigenetic inactivation of $p16^{INK4A}$ and $p14^{ARF}$ tumour suppressor genes are important role in CML progression.
2. We detected heterozygous point mutations in exon 1 of $p16^{INK4A}$ in 10 patients, in exon 2 in 11 patients and in exon 1 of $p14^{ARF}$ in 3 patients in the AP of CML.
3. Aberrant methylation of the $p16^{INK4A}$ or $p14^{ARF}$ promoters was found nearly in half of cases. Simultaneous methylation of both promoters was detected in 10 patients in our set of AP samples.
4. Two mutational hot spots of the *INK4A/ARF* locus have been detected. One of them was found at codon 35 (Ser to Ile) in exon 1 of $p16^{INK4A}$. The other mutational hot spot was located to codon 80 in exon 2 of $p16^{INK4A}$.
5. We found 1 heterozygous point mutation in exon 2 affecting both tumour-suppressor genes and 2 mutations in exon 1 of $p16^{INK4A}$ in BL cell lines.
6. We found the $p16^{INK4A}$ promoter to be completely or partially methylated while the $p14^{ARF}$ promoter was unmethylated in all EBV positive and negative BL lines. Both promoters were methylated in 4 of the 5 LCLs having surpassed 160 PDLs, but only the $p16^{INK4A}$ promoter in 1 "old" LCL. The status of both genes was normal in all *de novo* established LCLs, and there was no detectable methylation of the $p16^{INK4A}$ and $p14^{ARF}$ genes in normal B-cells.
7. Tumourigenesis of B-cells is a multistep process, the inactivation of INK4A/ARF tumour-suppressor genes occurring at a later stage of lymphomagenesis.
8. The methylation of $p16^{INK4A}$/$p14^{ARF}$ tumour suppressor gene may be an important hallmark of progression of tumour and immortalisation.

PUBLICATIONS

This thesis is based on the following publications:

1. Etelka Nagy, Zoltán Beck, Attila Kiss, Eszter Csoma, Béla Telek, József Kónya, Éva Oláh, Kálmán Rák, Ferenc D. Tóth: Frequent methylation of *p16^{INK4A}* and *p14^{ARF}* genes implicated in evolution of chronic myeloid leukemia from its chronic to accelerated phase.
   European Journal of Cancer, 39 (16), 2298-305 (2003) IF: 3.694

2. Etelka Nagy, György Veress, Krisztina Szarka, Eszter Csoma, Zoltán Beck: Frequent methylation of p16^{INK4A}/p14^{ARF} promoters in tumourigenesis of Epstein-Barr virus transformed lymphoblastoid cell lines
   Anticancer Research in press IF: 1.347

Other publications:

1. Eszter Csoma, Attila Bácsi, Xiangdong Liu, Judit Szabó, Peter Ebbesen, Zoltán Beck, József Kónya, István Andirkó, Etelka Nagy, and Ferenc D. Tóth: Human Herpesvirus 6 Variant A Infects Human Term Syncytiotrophoblasts In Vitro and Induces Replication of Human Immunodeficiency Virus Type 1 in Dually Infected Cells.
   Journal of Medical Virology, 67, 67-87 (2002) IF: 2.629

2. Zoltán Beck, Attila Bácsi, Xiangdong Liu, Peter Ebbesen, István Andirkó, Eszter Csoma, József Kónya, Etelka Nagy, and Ferenc D. Tóth: Differential Patterns of Human Cytomegalovirus Gene Expression in Various T-Cell Lines Carrying Human T-Cell Leukemia-Lymphoma Virus Type 1: Role of Tax-Activated Cellular Transcription Factors.
   Journal of Medical Virology, 71 (1), 94-104 (2003) IF: 2.371

3. Beck, Z., Bácsi, A., Nagy, E., Csoma, E. and Tóth, F.D.: Induction of full replication cycle of human cytomegalovirus by the Tax protein of HTLV-I in CD4+ T cells
   AIDS Res. Hum. Retroviruses 19, p:80 (2003) IF: 2,291

ORAL PRESENTATIONS, POSTERS

1. Nagy E., Beck Z., Csoma E., Bácsi A., D. Tóth F.: Az INK4A/ARF lókusz genetikai változásai a krónikus myeloid leukémia akcelerációjában. (Genetic alterations of INK4A/ARF locus in chronic myeloid leukaemia acceleration)
   Congress of Hungarian Society For Oncology, October 4-5, 2002. Kecskemét (presentation)

2. Nagy E., Beck Z., Csoma E., D.Tóth F.: Loss of $p16^{INK4A}$ protein expression in Epstein-Barr virus immortalized B-cells: Role of the LMP-1 protein
   14^{th} International Congress of the Hungarian Society for Microbiology, October 9-11., 2003., Balatonfüred (poster)

3. Nagy E., Beck Z., D. Tóth F.: Az INK4A gén metilációjának szerepe a krónikus myeloid leukémia progressziójában (Role of INK4A gene methylation in progression of chronic myeloid leukaemia)
   25^{th} Congress of Hungarian Society For Oncology, november 12-15, 2003., Szeged (presentation)

4. Nagy E., Beck Z., Csoma E., D. Tóth F.: A $p16^{INK4A}$ protein expresszió hiánya Epstein–Barr vírus által immortalizált B-sejtekben: Az LMP1 protein szerepe (Loss of $p16^{INK4A}$ protein expression in Epstein-Barr virus immortalized B-cells: Role of the LMP-1 protein)
   1^{st} Congress of the Hungarian Society for Molecular and Predictive Epidemiology, november 28-29, 2003. Pécs (presentation)

5. Nagy E., Csoma E., Beck Z.: A $p16^{INK4A}$ és $p14^{ARF}$ gének genetikai és epigenetikai változásai Epstein–Barr vírus által transzformált B-sejtvonalakban (Genetic and epigenetic alterations of $p16^{INK4A}$ and $p14^{ARF}$ genes in Epstein-Barr virus transformed B-cell lines)
   Congress of the Hungarian Society for Microbiology, October 7-9., 2004., Keszthely (presentation)

6. Beck Z., Csoma E., P. Ebbesen, Nagy E., D. Tóth F: Human Immunodefficiency Virus Type I Pseudotype With Envelope Antigens Of Human Cytomegalovirus Permissively Infects Human Syncytiotriphoblasts
   14th International Congress of Hungarian Society For Microbiology, Balatonfüred, October 9-11, 2003 (presentation)

7. Beck, Z., Bácsi, A., Nagy, E., Csoma, E. and Tóth, F.D.: Induction of full replication cycle of human cytomegalovirus by the tax protein of HTLV-I in CD4+ T cells
   11th International conference on human retrovirology, 2003, San Francisco, USA

8. Beck Z., Nagy E., Csoma E: Gyakori *p16*^{INK4A} és *p14*^{ARF} gén deléció, valamint promoter metiláció HTLV-I által fertőzött T-sejtvonalakban (Frequent deletion and promoter methylation of *p16*^{INK4A} and *p14*^{ARF} genes in HTLV-I infected T-cells)
   Magyar Mikrobiológiai Társaság Nagygyűlése, 2004. október 7-9., Keszthely (előadás)