**ELTE-TTK Biological Ph.D. School, Immunology subprogram**

# The role of the microRNA miR-146a in tumorigenesis and its mechanism of action

**(A miR-146a mikroRNS tumor-kialakítási szerepének és mechanizmusának vizsgálata)**

**Thesis of Ph.D dissertation**

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## INTRODUCTION

The regulation of gene expression is indispensable for the proper build up and function of cells. This co-regulated process occurs at multiple levels, its first step is transcriptional regulation. In vertebrates epigenetic mechanisms control promoter activity. These mechanisms include DNA methylation, histone modifications and certain processes regulated by small non-translated RNAs. Regulatory processes can't be understood without the analysis of transcription factors and signaling pathways.

Epigenetic regulatory mechanisms play a leading role in the control of gene activity, and they are of vital importance for the proper functions of the cell. For this reason, alteration of epigenetic regulation may result in pathologic changes and development of tumors. During my work, based on the analysis of the literature, I attempted to study the control processes regulating the expression of miR-146a, a microRNA that facilitates the generation of certain malignant tumors but inhibits the development of others. For a better understanding of the putative tumor-causing role of miR-146a and its mechanism of action, I used epigenetic methods and applied the tools of protein analysis as well. In healthy cells of the immune system, miR-146a plays an important role in various processes. It was demonstrated that the level of miR-146a changed in several pathologic alterations, and such a change may induce or initiate tumor development. LMP1, one of the proteins encoded by Epstein-Barr virus (EBV) controls miR-146a expression *via* the NFκB signal pathway. TRAF6 and IRAK, the proteins regulated by miR-146a, also belong to the very same signal pathway, forming thereby a complex regulatory circuit. A regulatory role for c-Myc, a cellular protein known to be connected to EBV was also suggested. Thus, in my experiments I used EBV positive and negative (LMP1 positive and negative) epithelial and B cell lines.

I found that the expression of miR-146a is regulated in a complex manner by several epigenetic mechanisms that act in concert. These results may help to elucidate the role of miR-146a in tumor development and its putative mechanisms of action, and may contribute, on the long run, to the development of novel therapies and preventive measures.

## AIMS

In order to assess the role of miR-146a in tumor development, I wished to overview the literature and study the following areas regarding the regulation of the miR-146a promoter:

(1) characterization of miR-146a promoter activity, detection of precursor and mature miR-146a RNAs in EBV positive cell lines;

(2) evaluation of the role and importance of DNA methylation in the regulation of the promoter, comparison of the data with the published findings;

(3) determining the role of histone modifications in promoter activity by analyzing 3 different regions of the miR-146a gene;

(4) evaluation of the concerted action of DNA methylation and histone modifications on promoter activity;

(5) analysis of transcription factor binding in the promoter region *in vivo*, based on factor binding sites predicted by *in silico* studies;

(6) evaluating the role of EBV in the regulation of miR-146a transcription;

(7) assessment of the influence of DNA methylation at the binding sites of c-Myc and NFkB with respect to promoter activity.

## MATERIALS AND METHODS

Cell lines

EBV positive and negative (LMP1 positive and negative) epithelial and B cell lines were used that provide a suitable model regarding the mechanisms of *in vivo* tumor development and maintenance.

Control sequencing

In order to perform the studies planned it was indispensable to determine the sequence of the gene regions analysed. For this reason I determined and compared with each other the target sequences in each cell line included into my experiments.

Analysis of gene expression

The goals of these investigations were:

- detection of precursor and mature miR-146a RNA forms in EBV positive and negative cell lines;
- comparison of miR-146a promoter activity in the cell lines studied, in order to evaluate the significance of the epigenetic regulatory mechanisms analyzed

Methods:

- Nuclear „run-on" analysis followed by real-time PCR was used for the determination of the level of the primary transcript. I used the method described by Patrone et al. (2000) with modifications.
- I used Northern blot to determine the level of the mature form of the RNA as described by Szittya et al., (2002) and Csorba et al., (2007), with modifications.

Analysis of DNA methylation

The methylation status of CpG dinucleotides located to the promoter region was determined using direct sequencing of bisulfite treated DNA samples. DNA was modified as described by Frommer et al. (1992) and Clark et al. (1994), with minor changes.

Analysis of histone modifications

I used chromatin immunoprecipitation, followed by real-time PCR to study the acetylation of histone 3 and histone 4 as well as methylation of lysine 4 in histone 3 within 3 regions of the gene. Chromatin immunoprecipitation was performed as described by Farnham et al., (2002), with minor changes.

DMS *in vivo* footprinting

Protein-DNA interactions were analyzed using DMS treatment followed by ligation mediated PCR and sequencing as described by Mueller and Wold, 1989, and Garrity and Wold, 1992, with modifications as in Niller et al., 1995, and Szenthe et al., 2013c.

## RESULTS AND DISCUSSION

In order to understand the role of the human microRNA miR-146a in tumorigenesis and its mechanism of action, it is indispensable to elucidate the mechanisms regulating its expression level.

The main results of my experiments were:

- the activity of the miR-146a promoter is better reflected in the amount of the primary transcript (precursor RNA) than in the amount of the mature form of miR-146a.
- The absence of DNA methylation is insufficient, in itself, to switch on the promoter. It is worthy to note that hypermethylation could be observed only in 2 in cell lines with a silent promoter, the other cell lines carried moderately methylated or unmethylated CpG dinucleotides in the regulatory region.
- Active promoters were invariably unmethylated.
- There was a medium or high level of the histone modifications analyzed at the active promoters, mainly in the regulatory region and the first intron. In contrast, in cell lines with silent promoters the level of modifications was either low or absent.
- As to the relationship of DNA methylation and histone modifications it is noteworthy that the activating modifications were nearly absent at the hypermethylated, silent promoters of certain BL lines and they were present in a low level in epithelial cell lines with a variable degree of promoter methylation.
- *In vivo* analysis of the *in silico* predicted factor binding sites of the miR-146a promoter demonstrated binding of c-Ets, c-Myc és NFkB in the analyzed miR-146a expressing BL cell line and the non-expressor epithelial cell line, but not in the BL cell line with silent miR-146a promoter.
- In BL cell lines, binding of transcription factors correlated well with promoter activity, whereas in case of the epithelial cell line other factors or the absence of modifications could possibly contribute to the inactivity of the promoter.
- PU.1 binding was not observed in the cell lines analyzed.

- Published data suggested a role for c-Myc and NFkB in the regulation of the promoter. Both factor binds in a methylation sensitive manner. I observed that the c-Myc binding site was unmethylated in almost all of the cell lines studied (there was a very weak methylation in one cell line), indicating that factor binding is possible. However, in BL lines not expressing miR-146a, a repressor chromatin structure may hinder c-Myc binding.

- Binding of NFkB was similar to that of c-Myc, with the exception of the inactive promoters carried by the cell lines Rael and Akata, where a high level methylation of the binding site may inhibit transcription factor binding.

- When evaluating the results, it is important to take into consideration the LMP1 positivity of the cell lines because LMP1increases the level of NFkB. We observed, however, a high or moderate level of miR-146a expression in certain EBV negative cell lines even in the absence of LMP1 expression. This suggests a role for other activator factors and mechanisms in the regulation of the promoter.

- The analysis of the BJAB cell line showed that a high miR-146a promoter activity may also occur in an EBV negative cell characterized by a low level of NFkB.

- The analysis of the C666-1 epithelial cell line demonstrated that binding of NFkB to the regulatory region of the miR-146a gene is insufficient for the activation of the miR-146a promoter.

## PUBLICATIONS IN CONNECTION WITH THE DISSERTATION

Szenthe K, Koroknai A, Banati F, Bathori Z, Lozsa R, Burgyan J, Wolf H, Salamon D, Nagy K, Niller HH, Minarovits J. (2013). **The 5' regulatory sequences of active miR-146a promoters are hypomethylated and associated with euchromatic histone modification marks in B lymphoid cells.** Biochem Biophys Res Commun. 433(4):489-95

Szenthe K, Nagy K, Buzas K, Niller HH, Minarovits J. (2013). **MicroRNAs as targets and tools in B-cell lymphoma therapy.** Journal of Cancer Therapy 4:466-474. DOI:10.4236/jct.2013.43A057

## PRESENTATIONS IN CONNECTION WITH THE DISSERTATION AND A PUBLICATION RELATED TO ANOTHER TOPIC

Szenthe K, Koroknai A, Banati F, Bathori Z, Helmut Niller H, Wolf H, Nagy N, Klein E, Minarovits J, Salamon D. (2013). **The role of DNA hypomethylation, histone acetylation and in vivo protein-DNA binding in Epstein-Barr virus-induced CD23 upregulation.** Biochem Biophys Res Commun. 435(1):8-15. doi: 10.1016/j.bbrc.2013.03.127.

Szenthe K, Bakos K, Bánáti F, Koroknai A, Niller HH, Minárovits J. (2011). **High resolution methylation analysis of the human CD40 promoter in Epstein-Barr virus (EBV) positive and negative cell lines.** Acta Microbiologica et Immunologica Hungarica, 58:(Suppl.) pp. 223-224.

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Szittya G, Molnár A, Silhavy D, Hornyik C, Burgyán J. (2002)**. Short defective interfering RNAs of tombusviruses are not targeted but trigger post-transcriptional gene silencing against their helper virus.** Plant Cell, 14(2):359-72.