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From reverse transcription to human brain tumors

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Reverse transcriptase from avian myeloblastosis virus (AMV) was the subject of the study, from which the investigations of the Department of biosynthesis of nucleic acids were started. Production of AMV in grams quantities and isolation of AMV reverse transcriptase were established in the laboratory during the seventies of the past century and this initiated research on the cDNA synthesis, cloning and investigation of the structure and functions of the eukaryotic genes. Structures of salmon insulin and insulin-like growth factor (IGF) family genes and their transcripts were determined during long-term investigations. Results of two modern techniques, microarray-based hybridization and SAGE, were used for the identification of the genes differentially expressed in astrocytic gliomas and human normal brain. Comparison of SAGE results on the genes overexpressed in glioblastoma with the results of microarray analysis revealed a limited number of common genes. 105 differentially expressed genes, common to both methods, can be included in the list of candidates for the molecular typing of glioblastoma. The first experiments on the classification of glioblastomas based on the data of the 20 genes expression were conducted by using of artificial neural network analysis. The results of these experiments showed that the expression profiles of these genes in 224 glioblastoma samples and 74 normal brain samples could be according to the Kohonen's maps. The CHI3L1 and CHI3L2 genes of chitinase-like cartilage protein were revealed among the most overexpressed genes in glioblastoma, which could have prognostic and diagnostic potential. Results of in vitro experiments demonstrated that both proteins, CHI3L1 and CHI3L2, may initiate the phosphorylation of ERK1/ERK2 and AKT kinases leading to the activation of MAPK/ERK1/2 and PI3K/AKT signaling cascades in human embryonic kidney 293 cells, human glioblastoma U87MG, and U373 cells. The new human cell line 293_CHI3L1, stably producing chitinase-like protein CHI3L1 was developed and these cells were found to have an accelerated growth rate and could undergo anchorage-independent growth in soft agar which is one of the most consistent indicators of oncogenic transformation. The formation of tumors in rats by 293_CHI3L1 cells evidences that CHI3L1 is an oncogene involved in tumorigenesis. In vitro experiments showed that constitutive expression of CHI3L1 gene promotes chromosome instability in 293 cells.

Keywords: reverse transcriptase, brain tumors, differential gene expression, chitinase-like proteins, CHI3L1 oncogene.

Some history. The beginning of the investigations which are carried out in the Department of biosynthesis of nucleic acids may be related to the early 70th when the reaction of reverse transcription was discovered [1, 2]. However, the scientists of the Institute of molecular biology and genetics were ready to accept this great discovery because already in 1961 Professor S. M. Gershenson hypothesized that the process of reverse transcription might exist in living organisms [3]. Unfortunately, at that time the Institute did not have any facilities to conduct such extraordinary sophisticated experiments in this field, so two scientists Alla Rynditch and Vadym Kavsan began the first experiments on synthesis of cDNA by AMV reverse transcriptase in the Institute of molecular biology (Moscow), first in the lab of Dr. R. Sh. Bibilashvili and then in the lab of Prof. V. A. Engelgardt. They were the first in the former Soviet Union, who synthesized the globin cDNA in 1974 and later reverse transcribed the messenger RNAs of mouse plasmocytoma [4] and in such a way put the first brick in the

organization of the International Project «Revertase-oncogene», which was the beginning of genetic engineering and biotechnology in the country. The main participants of the Project got the State Prize in 1979.

After returning to the Institute in Kiev they organized the second in the world (after the Bird and Bird's laboratory in the United States) unique laboratory on the production of avian myeloblastosis virus in grams quantities and isolation of AMV reverse transcriptase to supply it to many laboratories of different countries. The possession of very substantial amounts of this unique enzyme [5] allowed the scientists of the Institute to have a wide collaboration in different fields of molecular biology. Thus, they tried to find reverse transcription activity and discovered DNA-dependent DNA polymerase activity associated with *Galleria mellonella* L. nuclear polyhedrosis virus [6], performed comparative study on RNA-dependent DNA-polymerases (reverse transcriptases) of avian myeloblastosis and visna viruses [7], and showed the sequence homology between BSMV RNA species [8]. The development of new method for determination of poly(A)-sequences in RNAs with the help of reverse transcription [9] gave the opportunity to detect the polyadenilate sequences in RNA components of burley stripe mosaic virus [10].

We were the first who performed the DNA synthesis by AMV DNA polymerase on the heterogeneous nuclear RNA template [11] and on giant nuclear RNA [12]. In collaboration with Prof. G. P. Georgiev (Moscow) it was discovered and confirmed on the original models the ambiguous transcription boundaries in eukaryotes that allowed detecting a mechanism of the processed genes formation. It was shown for the first time that cDNA molecule, synthesized on pre-mRNA template, had a «lasso»-like structure [13–15] reflecting lasso-like form of RNA in splicing that was confirmed later by biochemical analyses. This finding was useful for the understanding of the pre-mRNA splicing mechanisms [16, 17].

Not only optimal conditions of the cDNA synthesis by AMV reverse transcriptase was established during the long period of the investigations [18–20] but AMV reverse transcription was also used as a model for screening and study of chemotherapeutic agents against retroviral infections, particularly 3'-asido-2',3'-dideoxythymidine which later was employed for the HIV1 treatment [21–23]. The new strain of HIV1 and the nucleotide sequence of its genome has been firstly described in Ukraine [24]. The structure of RSV adapted forms was determined that allowed to elucidate the mechanisms of the adaptation of oncogenic retroviruses to new hosts and to show that for the maintenance of tumorigenesis is not necessary to save a viral oncogene initiated this process proving the invalidity of oncogene addiction conception [25–28].

Several eukaryotic genes were synthesized by AMV reverse transcriptase and their structures were analyzed after cloning in plasmid vectors. Cloning of the rabbit globin cDNA and analysis of the globin-specific sequences in poly(A)-containing pre-mRNA from rabbit bone marrow erythoroid cells allowed to characterize the structure of globin-gene family as a model of eukaryotic gene structure [29–32]. Scientists of the department participated in the investigations on interferon genes and the construction of recombinant plasmids *pIFN-Ftrp* encoding synthesis of human leukocyte interferon cDNA with the aim of obtaining recombinant protein as therapeutic agent, first in the former Soviet Union [33, 34].

Insulin and insulin-like growth factors genes. The structures of salmon insulin-like growth factor I (*IGF1*) and insulin-like growth factor II (*IGF2*) genes and their promoter regions were determined after long-term investigations. Study on these genes, encoding growth-promoting peptides, as started from isolation and determination of nucleotide sequences of cDNA clones from salmon Brockman bodies cDNA library and as continued by the construction of salmon genome library and examination of the corresponding genomic clones [35–44]. Allelic polymorphism was described for insulin, *IGF1* and *IGF2* genes during analysis of the salmon genome [45–47]. For the first time it was shown that the salmon genome contained growth hormone pseudogene [48] as well as two insulin genes [46] and two insulin-like growth factor I genes; the second nonallelic *IGF1* gene was isolated from salmon genomic DNA library [47]. Salmon *IGF* genes and their promoters were investigated in details [38, 39]. It was revealed that the chum salmon IGF1 promotor is activated by hepatocyte nuclear factor 1 [40]. At the same time it was shown that *IGF2* promoter was activated by hepatocyte nuclear factor 3β [42] and requires Sp1 for its activation by C/EBPb [43].

Phylogenetic analysis of IGFs and their receptors was carried out on the basis of the obtained results and analysis of evolutionary conservation provided insights into the essential regions of molecules of these hormones and their receptors [49].

Searching for new glioblastoma markers. Investigations of tissue-specific genes expression were carried out by analysis of human liver and brain cDNA libraries at that time when Human Genome Project was started [50–53]. This study was transformed into investigations of the role of gene expression changes in the initiation and progression of brain tumors. Several dozens of genes differentially expressed in glioblastoma, the most aggressive form of the brain tumors, and normal brain were identified by differential hybridization of human brain cDNA library [54–57]. To identify the genes that might be used as molecular markers of glial tumors, gene expression in astrocytic gliomas of WHO grade II–IV and normal adult human brain was analyzed by Serial Analysis of Gene Expression (SAGE) [58, 59]. In our first work [58], the comparison of five glioblastoma (GB) SAGE libraries with two normal brain (NB) SAGE libraries, available at that time, has revealed 117 genes with more than 5-fold difference of expression levels in GB, $P \leq 0.05$.

Four new GB SAGE libraries appeared thereafter in the SAGE Genie database and the amount of other astrocytoma SAGE libraries were also increased. Thus, nine SAGE libraries of human glioblastoma (WHO grade IV astrocytoma, GB), eleven SAGE libraries of human anaplastic astrocytoma (WHO grade III, AA), eight SAGE libraries of human astrocytoma (WHO grade II, A) and five SAGE libraries of normal human brain (NB) were analyzed to compare gene expression in astrocytic gliomas with that of NB by accessing SAGE NCBI web site (http://www.ncbi.nlm.nih.gov/SAGE) and using the search tool of Digital Gene Expression Displayer (DGED) provided by the SAGE Genie database [59]. Comparison of the pools of 9 GB SAGE libraries and 5 NB SAGE libraries has revealed 129 genes with more than 5-fold differences in expression level compared to NB. 44 genes of 129 met the criteria for genes overexpressed in tumors. The number of genes with more than 5-fold differences in AA was 66 genes with 18 genes as overexpressed. 42 genes were shown as differentially expressed in diffuse astrocytoma, 16 of them increased their expression. Thus, the obtained results showed that the number of genes activated in astrocytic tumors was increasing during malignancy progression. Some expression changes occured early in astrocytoma formation and remained through passage to more malignant state, other changes were characteristic only of the most malignant stages of astrocytoma [59]. Northern blot analysis confirmed SAGE results in several arbitrarily selected differentially expressed transcripts. The expression patterns were usually reproducible between different samples. It is important to note, however, that there were differences in the gene expression levels between individual GBs [59]. Such differences in the gene expression undoubtedly contribute to the observed heterogeneity in the biological properties of cancers derived from the same organ [60, 61].

To enhance glioblastoma marker discovery we used DGED analysis of the pools of 9 GB SAGE-libraries and 5 NB SAGE libraries and revealed 676 genes, 316 of which were determined as overexpressed. To compare our SAGE results on the genes, which changed their expression in GB with those obtained by microarray technique, the expression factor 2 and significance filter $P \leq 0.05$ were chosen because these parameters were used mostly in the microarray analyses. Unfortunately, the comparison of even available data shows quite poor overlapping of the genes revealed by microarrays in different papers. The explanation of such significant differences in obtained results was given in four independent studies [66–69], which confirmed three persistent criticisms of the approach: the bewildering array of platforms and research protocols available make results from different studies hard to compare; in the hands of less experienced labs, homemade arrays are less dependable than commercial chips; different labs doing the same study can often get very different results. Comparison of our SAGE results on the genes overexpressed in GB with microarray analysis results revealed a limited number of common genes. Alltogether, 105 of 849 described genes were overlapping with those obtained by SAGE.

From our point of view, the main problem in evaluation of results obtained by comparison of gene expression in glioblastomas and normal brain samples was the lack of available data from each paper. The reason of poor overlapping of the genes revealed by microarrays

apparently is due to methodological artefacts (*e. g*. different gene numbers placed onto chips, poor quality of synthesized total cDNA probes or high background of hybridization patterns, problems with house-keeping gene controls, *etc*.) as well as to biological reasons (*e. g*. heterogeneity of molecular mechanisms of glioblastoma formation). A very big problem is obtaining normal brain samples. Usually, surgical specimens of histologically normal brain, adjacent to the tumor, are used as the source of normal brain RNA, however they can be considered as a normal control only with some precautions: gliomas are infiltrating tumors and scattered tumor cells are present far away from the dense tumor area removed during surgery. Apparently, the best solution of the problem in searching for GB markers is to compare all available results and to select only those genes, which significant expression in the tumor combined with no detectable or very low expression in normal tissues was reproduced in several articles. 105 differentially expressed genes, revealed by both methods, can be included in the list of candidates for the molecular typing of GB [62].

Some of overexpressed in glioblastoma genes may encode oncoproteins and some underexpressed genes may be the tumor suppressor genes. Thus, functional analysis of several identified differentially expressed genes was carried out to clarify the role of interaction of potential oncoproteins and tumor suppressor proteins with RAS/MAPK and PI3K/AKT signaling cascades, involvement of these interactions in the malignant transformation of brain cells, acquirement of proliferative and invasive properties by tumor cells. Products of overexpressed genes participate in angiogenesis, immunity, ECM, cell signaling pathways, and related to the IGF-system.

The genes of IGF-like family in glioblastoma. In recent years, the evidences have appeared that the members of IGF system may be involved in cancer development. Increased expression of *IGF1*, *IGF2*, their receptors, and binding proteins, or combinations thereof has been documented in various malignancies including gliomas. The results of multiple investigations suggest that the IGFs can play a paracrine and/or autocrine role in promoting tumor growth *in situ* during tumor progression but it may vary depending on the tissue of origin. Despite that the role of IGFs, IGF receptors, and IGF-binding proteins (IGFBPs) in tumor development is poorly understood up to this time, the antisense strategies, directed to the components of IGF-signaling, are the subject of many clinical trials. All three IGF receptors (IGF1R, INSR and IGF2R) are very well known targets for anti-cancer therapy. The increased expression of IGF1 receptor as well as its ligands may stimulate the PI3K and MAPK signaling cascades leading to cell proliferation.

We analyzed the expression of IGF system members including all ten IGFBP genes in glioblastoma by different methods to clarify their expression patterns in this tumor However, enhanced expression of the IGF1 gene in glioblastomas was not found when we used SAGE or analyzed data from the GEO repository [74]. Taking into account quite a big number of samples and different methods used in the present investigation, these results indicate that increased IGF1 gene expression might be involved in the formation of only limited part of astrocytic gliomas. Although the IGF1 was proposed as one of targets for glial tumor therapy and was supposed to become the alternative treatment of human glioblastoma [75], our results clearly showed why the anti-IGF1 treatment cannot give positive results with gliomas, supposing that the development of these tumors is activated by some other way. In contrast to IGF1, the expression of the *IGF2* gene is up-regulated in glioblastoma. The microarray analysis data showed the existence of separate group of the glioblastomas overexpressing the *IGF2* gene. This finding was in agreement with the results of Soroceanu *et al*. [76] who found that among 165 primary high-grade astrocytomas, 13 % of glioblastomas and 2 % of anaplastic astrocytomas expressed *IGF2* mRNA at the levels 50-fold higher than sample population median. IGF2 can substitute for EGF to support the growth of glioblastoma-derived neurospheres and growth-promoting effects of IGF2 were mediated by the IGF1 receptor and phosphoinositide-3-kinase regulatory subunit 3 (PIK3R3), a regulatory subunit of PI3K.

The results of the analysis of *IGFBP* genes expression in glioblastoma, obtained by three methods (SAGE, microarray analysis and RT-PCR), demonstrate up-regulation of the majority *IGFBPs* in this tumor. Produced in tumor cells, IGFBPs may stabilize insulin-like growth factor(s), IGF1 and/or IGF2, and drive their activation in glial tumors [74]. On the other hand, some of

the IGFBPs inhibit IGF actions or may act by a mechanism independent of IGFs, as reviewed by Mohan and Baylink [77].

Thus, increased expression of the IGF-binding protein genes in brain tumors makes the picture even more complicated. Our data highlight the importance of viewing the IGF-related proteins as a complex multifactorial system and show that changes in the expression levels of any one component of the system, in a given malignancy, should be interpreted with caution. As IGF targeting in anti-cancer therapy is rapidly becoming clinical reality, an understanding of this complexity is very timely.

The chitinase-like genes in glioblastoma. As it was described above, IGF1 is a key peptide in many tumors but was not found as overexpressed in glioblastoma [74], so it was supposed that IGF1 participation in the development of glial tumors may be substituted by protein products of other highly expressed genes, also participating in the PI3K and MAPK pathways. Chitinase-like glicoprotein CHI3L1 (other names YKL-40 and HC-gp39), encoding by the *CHI3L1* gene with considerably increased expression in most part of glioblastomas [78] could participate instead of IGF1 in the development of glioblastoma formation. It was shown that just as IGF1, it may stimulate the ERK1/2- and AKT-signaling pathways, associated with mitogenesis control, in a concentration range similar to the effective dose of IGF1 [79]. The new human cell line 293_CHI3L1 stably producing CHI3L1 was constructed and found that these cells had an accelerated growth rate and could undergo anchorage-independent growth in soft agar what is one of the most consistent indicators of oncogenic transformation [80, 81]. 293_CHI3L1 cells had activated PI3K and MAPK pathways; phosphorylated protein kinase B (AKT) was localized in cytoplasm while extracellular signal regulated kinases (ERK1/2) were localized in both cytoplasm and nuclei where they could activate different transcription factors with certain biological outcome. The *CHI3L1* gene knockdown by siRNA transfection gave noticeable CHI3L1 protein blockade (80–90 %) with significantly reduced *pERK1/2* and the colony-forming ability in soft agar of 293_CHI3L1 cells. The formation of tumors in rats by 293 cells expressing *CHI3L1* evidences that *CHI3L1* is an oncogene which is involved in tumorigenesis. This was the first animal model of human brain tumor which could be used for studying various biological properties of brain tumors in the immunocompetent animals [82]. The obtained results demonstrate that the activity of CHI3L1 mediated by pathways involved ERK1/2 and AKT plays a growth-promoting role and the overexpression of CHI3L1 is likely to be critical in the development of some tumors.

Other gene with considerably increased expression in glioblastoma identified by SAGE was *CHI3L2* (*YKL-39*) encoded 39 kDa chitinase-like protein that like CHI3L1 is a member of the 18 glycosyl hydrolase family [83]. Northern blot hybridization confirmed the data of SAGE for the majority of glioblastomas [84]. High homology of nucleotide and amino acid sequences of CHI3L2 and CHI3L1 suppose some identity of their functions [85]. However, western blot analysis did not show simultaneous production of CHI3L2 and CHI3L1 in glioblastoma and anaplastic astrocytoma samples that evidence the differences in functions of these homologous proteins [84]. CHI3L2 also induced phosphorylation of ERK1/ERK2 as CHI3L1 did. The results of *in vitro* experiments demonstrated that both proteins, CHI3L1 and CHI3L2, might initiate the phosphorylation of ERK1/ERK2 leading to the initiation of MAP kinase signaling cascade in human embryonic kidney 293 cells, human glioblastoma U87MG, and U373 cells [86, 87]. Activation of ERK1/2 by CHI3L2 was more prolong than by CHI3L1, and declined after 2 h only by ~ 30 % while after activation by CHI3L1 it declined approximately to a basal level. In contrast to the activation of ERK1/2 phosphorylation by CHI3L1 that lead to a proliferative signal (similar to the EGF effect in PC12 cells), activation of ERK1/2 phosphorylation by CHI3L2 (similar to NGF) inhibited cell mitogenesis and proliferation. The diversity in their functional activities could be explained, firstly, by the fact that native CHI3L1 is glycosylated at Asn60 while CHI3L2 is not a glycoprotein. Besides, CHI3L1 has a cluster of basic residues which can bind heparin; CHI3L2 has a different amino acid sequence in this site. Third, in the ligand-binding groove CHI3L1 has two tryptophan residues, in CHI3L2 these tryptophans are mutated to lysines which change the protein charge and hydrophobicity [88].

Growing body of evidence suggests that sustained activation and nuclear migration of AKT is implicated in controlling of differentiation and apoptosis in several

cell lines [89, 90]. The involvement of CHI3L1 and CHI3L2 proteins in activation of PI3K/AKT pathway and treatment of 293 cells by CHI3L2 resulted in fast increase of AKT phosphorylation, which continued for a long time. Similar sustained phosphorylation was observed also for U373 cells. The obtained data demonstrated prolonged activation of AKT by the CHI3L2 protein like to previously reported data for NGF in PC12 cells [89, 91]. In contrast to CHI3L2, CHI3L1-induced time course activation of AKT demonstrated substantially different result – incubation of the cells with CHI3L1 caused more transient activation of AKT. The phosphorylated form of AKT after incubation with CHI3L2 protein was detectable both in cytoplasm and nucleus as opposed to localization of phosphorylated AKT under CHI3L1 influence in the cytoplasm only. Overall, the results of AKT induction by CHI3L1 and CHI3L2 suggest that CHI3L1 potentially has a significantly different effect on the function of this kinase as compared to CHI3L2. The cellular receptors mediating the biological effects of CHI3L1 and CHI3L2 are not yet known but the activation of cytoplasmic signal-transduction pathways suggests that these chitinase-like proteins interact with one or several signaling components on the plasma membrane. Different results of these interactions, revealed for CHI3L1 and CHI3L2, may have various impacts on the fate of the cells.

Glioblastoma treatment. Despite revealed *in vitro* anti-proliferative effect of the CHI3L1 oncoprotein suppression, therapy against one oncogene target cannot be effective and in the present decade it is believed that cancer therapy is going to shift slowly from «one target» to a more personalized multitarget approach. High heterogeneity of glial tumors makes necessary simultaneous analyses of many genes and therapy targeted not to individual genes, but to the physiological effect caused by these genes. Angiogenesis is an important part of the tumor development, through which the nutrients get into the centre of tumor developed in hypoxic conditions. This allows the cells of malignant neoplasms to proliferate under increased oxygenation conditions and removal of metabolic wastes which usually induce necrosis. When the number of tumor cells reaches a critical level, they contribute to the formation of new blood vessels and metastasis. A number of angiogenic factors such as VEGF and PDGF, play a key role in tumor vascularization. In anticancer therapy, a considerable attention paid to the anti-angiogenic drugs, which are approved by FDA, such as bevacizumab (antibodies against VEGF) and tyrosine kinase inhibitors of VEGF receptor (sorafenib and sunitinib). However, the success of these angiogenic drugs is a temporary one, the drug resistance, tumor recurrence and rapid firmation of the new blood vessels are developed at the end of the therapy. Besides, the opposite effect of angiogenic agents on tumor growth takes place, as well as on the angiogenesis and metastasis formation in xenograft tumor models as well. Oncogenic redundancy is a significant obstacle to the success of against targeting treatment. It was shown that CHI3L1 possessed highly proangiogenic properties [92]. So, simultaneous treatment with anti-CHI3L1 (as specific siRNAs) and anti-VEGF (as Bevacizumab) preparations may give positive results.

Of course, an obvious success may be predicted for complex cancer therapy with chemical preparations of different types. The study was initiated by evaluation of anticancer activity in compounds with distinct chemical nature, namely bradykinin (BK) antagonists and azolidinones-related chemicals, in several types of malignantly transformed cells: 293 cells, stably transfected by *CHI3L1* oncogene (293_CHI3L1), glioblastoma-derived U373 cells and mantle cell lymphoma (MCL) cell lines Granta, JeKo, Mino and UPN1. Nonapeptides BK possess many different activities related to normal physiology as well as to pathophysiology, namely the modulation of vascular tone, pain, and inflammation. BK has been shown to have growth-factor properties in human cancers of lung, prostate, ovarian, gastrointestinal, and breast, promotes the migration of glioma cells [93]. This formed the basis for development of new drugs, such as BK antagonists. We showed that several bradykinin antagonists have significant growth suppressor activity in 293_CHI3L1 and U373 cells and strongly inhibited extracellular signal-regulated kinases 1/2 (ERK1/2) and protein kinase B (AKT1) phosphorylation. Azolidinones are of great importance in modern medicinal chemistry and have been investigated for a range of pharmacological activities such as anti-inflammatory, antimicrobial, antiviral, antiproliferative, *etc*. Special attention was attracted to azolidinones as potential novel anticancer agents. Previously, the group of Dr. Roman Lesyk at Danylo Halytsky Lviv National Medical

University reported about growth-suppression activity of azolidinone derivatives, particularly against glioblastoma cell lines [94]. We found that one of examined preparations demonstrated high anti-proliferative properties. Thus, a growth suppression activity of two different classes of molecules was shown in three types of malignantly transformed cells. Our preliminary results demonstrated that molecular mechanisms of their action might rely on the modulation of key cellular signaling pathways. Further investigations of molecular mechanisms of BK antagonists and azolidinone derivatives action and pre-clinical studies using animal models are needed for the evaluation of these compounds as new anti-cancer drugs.

The description of new molecular markers is necessary for identification of specific gene expression profiles (signatures) in tumor cells which will be useful for understanding the molecular mechanisms participating in the arising and development of neoplasms as well as for determining the strategy of anticancer therapy. We made first attempts to develop such signature for glioblastoma. Neural network analysis was used for the classification of glioblastomas based on the data of 20 differentially expressed genes revealed by microarray analysis. The obtained results showed that the expression profiles of these genes in 224 glioblastoma samples and 74 normal brain samples can be clustered using the Kohonen's maps [95]. Subsequent comparison of the microarray analysis and SAGE results showed that about 30 differentially expressed genes are suitable for the recognition of specific gene expression profiles in glioblastomas and normal brain by artificial neural network.

For drugs delivery into the brain we develop the nanocojugates which should penetrate across the blood-brain and tumor-normal brain barriers. A therapeutic agent of created nanoconjugates is a morpholino antisense-oligonucleotide or siRNA to *CHI3L1* mRNA and some other agents. As a vector is used a polymer matrix presenting natural biopolymer poly-beta-maleic acid (β-L-malic acid, PMLA) from the microorganism *Physarum polycephalum*, which was isolated and transfer red to us by Dr. J. Lyubimova (Cedars-Sinai Medical Center, USA). Previously, the polyfunctional nanoconjugate Polycefin was developed in Dr. J. Ljubimova lab (Polycefin, US patent 2007/0259008 A1) and its inhibitory effect was demonstrated on the tumor growth in the brain of «nude» rats by inhibition of laminin-8 vascular protein overproduction in glioblastoma cells [96]. In our experiments, antisense-oligonucleotide to the *CHI3L1* mRNA will be attached to PMLA polymeric matrix via disulphide bonds which have to be disrupted in cytoplasm to release therapeutic agent(s) within cells. In addition to the therapeutic agent, several modules have been introduced into nanoconjugates required for directed delivery to the tumor cells as polyethylene glycol (PEG) to protect conjugate against rapid degradation, trileucine peptide for pH-dependent lipophilicity provision to destruct the endosomal membranes, antibodies against transferrin receptor (TfR) for getting into tumor cells and receptor-mediated endocytosis, reporter fluorescent dye for therapeutic detection (if necessary). It is expected that nanoconjugates of antisense-oligonucleotides or siRNA to *CHI3L1* mRNA with PMLA will have antiproliferative effect and will inhibit tumor cells growth due to suppression of CHI3L1 protein production in. Developed by us the new model of brain tumor in immunocompetent adult rats will be used to test these nanocojugates *in vivo* [82].

Cancer cells karyotyping and evolution of cancer. Chromosome instability (CIN) and the resulting clonal/non-clonal intratumor heterogeneity elucidate why large-scale tumor genome sequencing and high-resolution analysis of somatic copy-number alterations have failed to reveal «universal» cancer genes except well known for decades, and type- and stage-specific recurrent aberrations in solid tumors, whereas most recurrent chromosome aberrations (deletions, amplifications, and translocations) ever occurring genome-wide in tumors can be explained by 3D genome organization, spatial proximity among chromosome loci, and replication timing of sites producing rearrangements. CIN explains how mutagenic and non-mutagenic chemical agents, physical factors, contacts with bacterial cells, and infection with some viruses induce or promote transformation of cells *in vitro* and tumor development *in vivo*, as well as spontaneous *in vitro* transformation of primary and immortalized cells and tumorigenicity of induced pluripotent stem (iPS) cells. CIN accounts for the acquisition of oncogene independence and tumor recurrence after inductor withdrawal in oncogene on/off conditional transgenic mice models. CIN and intratumor heterogeneity are the reasons of onco-

gene addiction independence of solid tumors from any particular oncogene and general ineffectiveness of targeted therapy in clinic. Any factors or stresses that contribute to CIN can potentially promote the evolution of cancer (reviewed in [97]).

The process of cellular transformation has been amply studied *in vitro* using immortalized cell lines. Immortalized cells never have the normal diploid karyotype, nevertheless, they cannot grow over one another in cell culture (contact inhibition), do not form colonies in soft agar (anchorage-dependent growth) and do not form tumors when injected into immunodeficient rodents. All these characteristics can be obtained with additional chromosome changes. Multiple genetic rearrangements, including whole chromosome and gene copy number gains and losses, chromosome translocations, gene mutations are necessary for establishing the malignant cell phenotype. Most of the experiments detecting transforming ability of genes overexpressed and/or mutated in tumors (oncogenes) were performed using mouse embryonic fibroblasts (MEFs), NIH3T3 mouse fibroblast cell line, human embryonic kidney 293 cell line (HEK293), and human mammary epithelial cell lines (mainly HMECs and MCF10A). These cell lines have abnormal karyotypes and are prone to progress to malignantly transformed cells. The mechanisms of cell immortalization by different «immortalizing agents», oncogene-induced cell transformation of immortalized cells and moderate response of the advanced tumors to anticancer therapy in the light of tumor «oncogene and chromosome addiction», intra/intertumor heterogeneity, and chromosome instability are just discussed in review [98]).

For decades the conventional gene mutation cancer theory has been postulating that cancer is a genetic disease considered as a result of deterministic sequential accumulation of the mutations in handful of «driver» cancer genes occurring in a continuous linear pattern of cancer progression. However, in contrast to this postulate, the recent whole genome and exome sequencing studies of primary tumor bulk and metastases or separate regions within the same sample have revealed a large number of stochastic gene mutations for each individual with the same cancer type and significant intratumoral genetic heterogeneity with «branched evolutionary tumor growth» or «punctuated clonal evolution without observable intermediate branching» or «no dominant clones in the cancer tissue». Meanwhile, the stochastic karyotypic variation and intratumor heterogeneity are recognized to be the driving force of tumor evolution and major factors of recurrent tumors occurrence with acquired drug resistance. The karyotype evolution/chromosome instability and the resulting magnitude of intratumor heterogeneity significantly correlate with tumorigenic potential of cells, tumor disease progression from precancerous lesions to malignant tumors and metastases, correlate with patient survival, treatment sensitivity, and the risk of acquired resistance. We discuss importance of the evolutionary karyotypic theory in understanding of the cancer biology and mechanisms of tumor drug resistance [99].

Recently we have revealed that constitutive expression of CHI3L1 promotes chromosome instability in 293 cells. Modal number of chromosomes in 293_CHI3L1 cells is distinct to that in transfection control *293_pcDNA3.1* cells and parental 293 cells. Interline whole chromosome heterogeneity is manifested. A number of new distinct marker chromosomes were observed in CHI3L1-expressing cells from two independent experiments. Array comparative genome hybridization (aCGH) was used to analyze the subchromosomal alterations in these cell lines. The spectrum of cytoband gains and losses in 293_CHI3L1 cells was significantly different from control cells. Thus, we established the link between transforming properties of oncogene *CHI3L1* and changes of karyotype of 293 cells with stable expression of CHI3L1.

Conclusions. Reverse transcription and corresponding enzymes played a key role in the development of the investigations in the Department of biosynthesis of nucleic acids (IMBG, NANU). Similar projects became the most rapidly growing fields in molecular biology of 70–80 years of the last century. The possession of very substantial amounts of this unique enzyme gave opportunity for the fast creating of cDNA libraries on mRNAs of different origin, isolation of respective cDNA clones, determination of primary structure of different eukaryotic genes, and study on the structure of corresponding genome loci. Determined structures of the salmon insulin and insulin-like growth factor (IGF) family genes, their allelic polymorphism, promoters, and their transcripts became classical in the investigations of non-mammalian genomes.

The most suitable modern methods for determination of genes differentially expressed in astrocytic gliomas and human normal brain, SAGE and microarray hybridization analysis based on cDNA synthesis were used for identification of new glioma markers. Differentially expressed genes, common to both methods are the candidates for the molecular typing of glioblastoma.

It was found that overexpressed in glioblastomas the *CHI3L1* gene encoding chitinase-like protein CHI3L1 had oncogenic properties. Strategies, based on the complex therapy including inhibition of *CHI3L1* expression by nanocojugates of Morpholino antisense oligonucleotide to the *CHI3L1* mRNA and polymalic acid, will be used for the developing of the brain tumors therapy.

In vitro experiments showed that the constitutive expression of CHI3L1 gene promotes chromosome instability in 293 cells. Modal number of chromosomes in 293_CHI3L1 cells differs from that in transfection control *293_pcDNA3.1* cells and parental 293 cells. A number of new distinct marker chromosomes were observed in CHI3L1-expressing cells from two independent experiments. Thus, the link between transforming properties of oncogene *CHI3L1* and changes in karyotype of 293 cells, stably producing CHI3L1 protein, was established.

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Від зворотної транскрипції до пухлин головного мозку людини

Резюме

Наукові розробки відділу біосинтезу нуклеїнових кислот розпочато з вивчення зворотної транскриптази вірусу пташиного мієлобластозу (AMV). Протягом сімдесятих років минулого століття у відділі налагоджено виробництво AMV (декілька грамів на рік) та виділення зворотної транскриптази AMV, що дозволило розгорнути роботи з синтезу кДНК, клонування та вивчення структури і функції генів евкаріотів. Упродовж багаторічних досліджень було визначено будову генів інсуліну і родини інсуліноподібних факторів росту (IGF) лосося та їхніх транскриптів. Результати застосування двох сучасних методів – гібридизації мікрочіпів і SAGE – використано для ідентифікації генів, які диференційно експресуються в астроцитарних гліомах і нормальному головному мозку людини. Їхнє порівняння виявило обмежену кількість спільних генів, надекспресованих у гліобластомі. Визначені нами 105 диференційно експресованих генів, спільних для обох методів, можуть бути включені до переліку кандидатів для молекулярного типування гліобластом. Проведено перші експерименти з класифікації гліобластом на основі даних по експресії 20 генів із застосуванням штучної нейронної мережі, які показали, що профілі експресії зазначених генів для 224 зразків гліобластом і 74 зразків нормального головного мозку піддаються кластеризації згідно з картами Кохонена. Серед найекспресованіших у гліобластомі генів, які мають прогностичний і діагностичний потенціал, виявлено гени хітиназоподібних білків CHI3L1 і CHI3L2. Результати експериментів in vitro продемонстрували, що обидва білки – CHI3L1 і CHI3L2 – здатні ініціювати фосфорилювання кіназ ERK1/ERK2 і AKT, що спричиняє активацію сигнальних каскадів PI3K/AKT і MAPK/ERK1/2 в клітинах 293 ембріональної нирки людини, а також у клітинах U87MG і U373 гліобластоми людини. Ідентифіковано нову клітинну лінію людини 293_CHI3L1, яка стабільно продукує хітиназоподібний білок CHI3L1. Знайдено, що ці клітини мають прискорений ріст і можуть рости у м'якому агарі незалежно від прикріплення до поверхні, що є одним із найсуттєвіших показників пухлинної трансформації. Формування пухлин клітинами 293_CHI3L1 у щурів свідчить про те, що CHI3L1 є онкогеном, причетним до канцерогенезу. Експерименти in vitro засвідчили, що конститутивна експресія гена CHI3L1 сприяє хромосомній нестабільності у клітинах 293. Модальне число хромосом у клітинах 293_CHI3L1 відрізняється від такого хромосом у контрольних клітинах 293_pcDNA3.1, трансфікованих «порожнім» плазмідним вектором, і батьківських клітинах 293.

Ключові слова: зворотна транскриптаза, пухлини головного мозку, диференційна експресия генів, хітиназоподібні білки, онкоген CHI3L1.

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От обратной транскрипции к опухолям головного мозга человека

Резюме

Научные разработки отдела биосинтеза нуклеиновых кислот начались с изучения обратной транскриптазы вируса птичьего миелобластоза (AMV). В течение семидесятых годов прошлого века в отделе налажено производство AMV (несколько граммов в год)

и выделение обратной транскриптазы AMV, что позволило развернуть работы по синтезу кДНК, клонированию и исследование структуры и функции генов эукариотов. На протяжении многолетних исследований было определено строение генов инсулина и семейства инсулиноподобных факторов роста (IGF) лосося и их транскриптов. Результаты применения двух современных методов – гибридизации микрочипов и SAGE – использованы для идентификации генов, дифференциально экспрессируются в астроцитарных глиомах и нормальном головном мозге человека. Их сравнение выявило ограниченное число общих генов, надэкспрессированных в глиобластоме. Определенные нами 105 дифференциально экспрессированных генов, общих для обоих методов, могут быть включены в список кандидатов для молекулярного типирования глиобластом. Проведены первые эксперименты по классификации глиобластом на основе данных по экспрессии 20 генов с использованием искусственной нейронной сети, показавшие, что профили экспрессии этих генов для 224 образцов глиобластом и 74 образцов нормального головного мозга могут быть кластеризованы в соответствии с картами Кохонена. Среди наиболее надэкспресированных в глиобластоме генов, имеющих прогностический и диагностический потенциал, обнаружены гены хитиназоподобных белков CHI3L1 и CHI3L2. Результаты экспериментов in vitro продемонстрировали, что оба белка – CHI3L1 и CHI3L2 – могут инициировать фосфорилирование киназ ERK1/ERK2 и AKT, приводящее к активации сигнальных каскадов PI3K/AKT и MAPK/ERK1/2 в клетках 293 эмбриональной почки человека, а также в клетках U87MG и U373 глиобластомы человека. Получена новая клеточная линия человека 293_CHI3L1 со стабильной продукцией хитиназоподибного белка CHI3L1. Обнаружено также, что эти клетки обладают ускоренным ростом и могут расти в мягком агаре независимо от прикрепления к поверхности. Такие свойства являются одним из наиболее существенных показателей опухолевой трансформации. Формирование опухолей клетками 293_CHI3L1 у крыс свидетельствует о том, что CHI3L1 является онкогеном, участвующим в канцерогенезе. Эксперименты in vitro показали, что конститутивная экспрессия гена CHI3L1 способствует хромосомной нестабильности в клетках 293. Модальное число хромосом в клетках 293_CHI3L1 отличается от такового хромосом в контрольных клетках 293_pcDNA3.1, трансфецированных «пустым» плазмидным вектором, и родительских клетках 293.

Ключевые слова: обратная транскриптаза, опухоли головного мозга, дифференциальная экспрессия генов, хитиназоподобные белки, онкоген CHI3L1.

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