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Clinical aspects of molecular biology of pituitary adenomas

Dawid Larysz[1], Sławomir Blamek[2], Adam Rudnik[1]

[1]Department of Neurosurgery, Medical University of Silesia in Katowice, Poland, [2]Department of Radiotherapy, Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology, Gliwice Branch, Poland

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Abstract

Pituitary adenomas are primary, benign CNS tumors. Sporadically, they metastasize or become malignant. However, they can infiltrate adjacent structures even if they are benign and without hormonal activity. Moreover, by compressing adjacent tissues they cause their gradual degradation and, as a result, irreversible CNS damage. Pure endoscopic transnasal transsphenoidal approach enables minimally invasive resection of the aforementioned tumors. In most cases, standard total resection is sufficient but in some cases tumors could be recurrent. There are still unknown risk factors leading to recurrence and subsequent progression of these tumors. This is the reason why pituitary adenomas are a serious clinical and social problem in spite of their benign histology. Continuous development of immunohistochemical and proteomic examinations and application of advanced methods of functional genomics allow for better understanding of biology and pathogenesis of these tumors. In the paper authors discuss molecular etiopathogenesis of pituitary adenomas.

Key words: *pituitary adenomas, functional genomics, epigenetics, molecular biology.*

Pituitary adenomas are primary, benign CNS tumors. Sporadically, they metastasize or become malignant. In 5-35% of cases, however, they may infiltrate the adjacent structures, such as bony parts of the sella turcica, the sphenoid sinus, the dura mater lining the sella turcica, the cavernous sinuses, or the nervous tissue, penetrating inside the fourth cerebral ventricle and the hypothalamus. Moreover, by compressing adjacent cerebral structures they may cause their gradual degradation and result in irreversible CNS damage. Pituitary adenomas are revealed in about 14-27% of autopsies [14,21]. The morbidity, however, is estimated at about 10-17% [6,21], some authors cite the prevalence of 1 in 1064 people [15,64]. Since a vast majority of tumors identified in post mortem examinations do not exceed 10 mm in diameter, they are classified as microadenomas. This category of tumors in general tends to be asymptomatic. Systematically evolving imaging techniques, including magnetic resonance imaging, allow for detection of asymptomatically developing pituitary neoplasms during an examination carried out due to indications other than the diagnosis of the pituitary gland. The prevalence of adenomas revealed by such incidental diagnosis is estimated at about 22.5% [21,26,47]. Continuous development of immunohistochemical and proteomic methods, as well

Communicating author:
Dawid Larysz, MD, PhD, MSc, Dept. of Neurosurgery, Medical University of Silesia in Katowice, ul. Medyków 14, 40-752 Katowice, phone +48 32 789 45 03, fax +48 32 292 89 25, e-mail: dawilar@gmail.com

as the use of advanced methods of functional genomics, allow nowadays for a better understanding of both the pathogenesis mechanisms and the biology of these tumors [30,36,41,42,48,49,51,65,66].

Molecular pathogenesis of pituitary adenomas

The research carried out over the past 20 years has come up with two alternative theories of pathogenetic mechanisms. The first theory implies that pituitary adenomas result from a continuous, pathologic stimulation by factors regulating cell activity. These may be stimulating and suppressing substances produced by the hypothalamus, hormones secreted by endocrine glands, but also growth factors acting through auto- and paracrine feedback mechanisms. According to the other, recognized by the majority of researchers, there occur primary defects of hypophyseal cells. Overlapping and a synergistic action of both mechanisms is also considered probable [2,45].

The hypophysis–hypothalamus complex integrates excitatory and inhibitory stimuli of both central and peripheral origin. Synthesis and secretion of anterior pituitary hormones take effect in five specialized cell types which show expression of highly specific receptors for certain regulatory molecules. These receptors, after binding a specific ligand, regulate the process of production and secretion of anterior pituitary hormones by G-protein cascade. It is important to note that both central and peripheral regulatory agents can cause hyperplasia, involution or even neoplastic transformation of secretory cells [22]. *In vitro* studies revealed that in some cases stimulated hypophyseal secretory cells could gain mitotic activity in spite of a completed differentiation process. This kind of activity may occur e.g. during pregnancy [37,38,40]. Such situation, however, is very rare and it should be assumed that the hyperstimulation of hypophyseal cells by regulatory agents tends to increase the probability of new mutation or proliferation of previously mutated cells, rather than cause neoplastic transformation *per se* [40].

Pituitary secretory cells have, therefore, two mechanisms of activity. They respond to central and peripheral stimuli by secreting hypophyseal hormones, thus controlling broadly understood body homeostasis. On the other hand, being under the continuous influence of autocrine and paracrine feedback mechanisms, they are involved in a process called the pituitary plasticity. This process is characterized not only by a varying level of hormone secretion, but also morphological changes of the cells, or even their proliferation and dedifferentiation [17,44]. Pathologic proliferation of previously differentiated secretory cells invariably results in impairment of hormonal equilibrium and hyper- or hyposecretion of relevant hormones [17,45,46].

Aberrations leading to a pathologic development and maturity of the pituitary gland and, consequently, to an increased probability of a neoplastic transformation therein, often take place early in the fetal life. There exist a whole range of transcription factors produced in the midbrain and partially in the infundibulum, responsible for normal formation and maturation of central areas of the developing brain. Mutations or abnormal expression of genes encoding these factors (such as e.g. *RPX, LHX3, LHX4,* or *PITX2*) may cause pathologic maturation of central areas of the brain and result in pituitary hypoplasia. If some secretory cells reach their proper functional status anyway, peripheral over-stimulation by the feedback mechanism can promote formation of an adenoma in the hypoplastic pituitary [45]. Not all factors promoting hypoplastic pituitary can, however, be involved in the process of adenoma formation. Transcription factors, such as Pit1, Prop1, SF1, Tpit, which control the development of pituitary cells may be the cause of impaired secretion of pituitary hormones as well as of a hypoplastic pituitary when mutation has occurred in the genes encoding these factors. Such mutations, however, generally do not seem to be involved in neoplasia. Moreover, the presence of a normal expression of *PROP-1* and the absence of its mutations in adenomas may point to that gene as a crucial player for maturation of hypophyseal cells [44,45]. A previously mentioned possible role of caudal-related homeo-box transcription factor CDX2 in formation of pituitary adenomas was not confirmed [56].

Studies on the mechanisms of adenoma formation have proved a vast majority of them to be monoclonal tumors [12,32,45]. The tumor forms from a single, mutated cell which gains the ability to proliferate. The neoplastic transformation process is usually a multi-step cascade. It affects proliferation, differentiation (or dedifferentiation), as well as hormone synthesis and secretion processes. As for any other tumor, the direct cause of a neoplastic transformation of a hypophyseal secreting cell can either be improper oncogene activation or suppressive gene inactivation. It seems also possible for these two mechanisms to occur simultaneously. Nonetheless, the activation of oncogenes

seems a more primary process [10,45,48]. It should be noted, however, that monoclonality does not rule out the possibility of occurrence of cells of various morphology and biology within one tumor. As long as the malignancy arises from one abnormal cell, it is a truly monoclonal one. If, however, it forms on the basis of e.g. hyperplasia of specific cells differing from one another, each of them may be a source of an entire line of monoclonal cells, yet the tumor as a whole will contain different cells. This variability may affect the exact pathogenesis, the frequency of divisions, the type of variation or the increase in apoptosis. Furthermore, these cells are bound to respond differently to the same stimuli, such as pituitary specific oncogenes, growth factors or extra-pituitary tropic factors [12].

Expression of a single mutated oncogene allele is sufficient to cause impaired cell function. Such kind of mutation was revealed in 40% of somatotropinoma-type pituitary adenomas, in 10% of non-functioning adenomas (NF), as well as in 6% of adrenocorticotropinomas [24,45]. In these tumors, a single mutation in the *GNAS* gene, encoding GS-α, causes inactivity of GTP-ase (one of G-protein subunits). Subsequently, growth hormone secretion rises as a result of an increased cAMP concentration. Moreover, due to the impairment of the metabolic cascade initiated by the activation of the G-protein by the receptor, the growth hormone secretion is in these cases somatropin-independent [18,24,45,60]. A very similar mechanism is probably responsible for acromegaly in McCune-Albright syndrome [62]. It should be noted at this point that *GNAS* mutations are the only ones frequently encountered in sporadic pituitary adenomas.

Cyclin D1 is one of a group of proteins involved in cell-cycle regulation. Locus of its gene is localized in chromosome 11q13. According to Farell and Clayton, cyclin D1 gene amplification is a common finding in NF adenomas and infiltrating pituitary tumors [24]. In Tani's research, the level of expression of cyclins B1, D1 and E1 (but not cyclin A1) was decreased in adenomas resulting in Cushing disease, as compared to the so-called "silent-corticotroph" and NF adenomas. The same study revealed a four times higher expression of the *CDKN2A* gene in ACTH-secreting tumors which cause Cushing disease [61]. Similarly, an amplification of the H-RAS oncogene localized in 5p13 locus is responsible for the aggressive phenotype of prolactin-secreting adenomas and pituitary adenocarcinomas [22,23]. Nearly 1/3 of adenomas, either those secreting GH, PRL, and ACTH, as well as NF adenomas, are characterized by the overexpression of the *c-myc* oncogene localized in 8q24 [22,23,45,63].

Inactivation of suppressor genes seems to be a secondary mechanism of adenoma formation. Both alleles of the suppressor gene have to be mutated to induce neoplasia. These mutations may be either deletions or gene silencing (methylation) of a relevant DNA chain, or point mutations resulting in the loss of heterozygosity (LOH) [44,46].

As opposed to sporadic pituitary adenomas, where underlying germ-line mutations are very rare, in multiple endocrine neoplasia syndrome type 1 (MEN 1) a specific germ-line mutation predisposing to the disease has been revealed. The *MEN 1* gene is localized in 11q13 chromosome [35,50,59]. The gene encodes a nuclear protein, Menin, whose function includes the control of transcription and cell division, as well as cellular proliferation [1]. Adenomas occur in 40% of patients with MEN1 mutation. PRL-secreting tumors are the most common ones, while GH-secreting and NF tumors are also found, and ACTH-secreting ones are the most rare of all [16,65]. Adenomas in MEN patients tend to be more aggressive and prone to infiltrate the surrounding tissues than the sporadic ones [65].

Highly infiltrative tumors seem to be a separate group of pituitary adenomas in terms of molecular biology. It seems probable that the loss of heterozygosity (LOH) in chromosomes like 1, 11, 10, 13 may be associated with an increased potential to cellular invasion [7,23,58].

Transcription factors are also involved in the adenoma formation process. For instance, PIT-1 transcription factor mRNA level in pituitary adenomas secreting growth hormone and/or prolactin, is five-fold higher as compared to normal hypophyseal cells, which can be a result of either overexpression or amplification of *PIT1* gene. The gene expression product is identical as in normal hypophyseal cells [45,52,53]. Probably, several other transcription factors like PROP-1, CDK-4, ER-α, c-FOS, and c-MYC are involved in pituitary adenoma pathogenesis, along with kinases such as e.g. CDK-4 [45,49,59]. Another hypothesis, based on an impaired response of hypophyseal cells to a normal hypothalamic stimulation can also be of interest. It has been postulated that defective receptors for hypothalamic tropic factors can be expressed in hypophyseal cells. Also, hypo- or hyperexpression of normal receptors on cellular membranes, or impaired signal transduction have been deemed probable factors influencing adenoma formation [45]. The above hypo-

theses still await the experimental confirmation. It seems, however, that pituitary response to hypothalamic stimulation may play a significant role in the initial phase of neoplastic transformation.

The research on the pathogenesis of pituitary adenomas has taken into account also numerous "classic" epithelial growth factors and their receptors such as FGF and FGFR, VEGF and VEGFR, or EGF and EGFR, as well as other endo- and paracrine regulators of neoplastic cells, such as IL-6, IL-8 or IL-11 [27,28, 66,67,71].

Functional genomics investigations

Advanced methods of functional genomics have recently provided additional interesting insights into the understanding of molecular biology of pituitary tumors. Research based on oligonucleotide or cDNA microarrays allows for analysis of the expression profile of thousands of genes simultaneously, and the plethora of statistical tools like clustering or supervised gene selection helps to identify groups of transcripts with a similar expression, and subsequently find out which metabolic or signaling pathways are active in the cell. Evans *et al*. analyzed 32 adenoma specimens. The expression profile was investigated with cDNA microarrays capable of analysis of over 7 thousand genes. The obtained results allowed to distinguish separate expression profiles for PRL-, GH-, ACTH-secreting adenomas, and NF ones, as well as normal hypophyseal cells. NF adenomas showed a specific expression for 60 gene signature. Overexpression of 25 genes was detected, including a major overexpression of folic acid receptor gene (FR-α). Folic acid is a vitamin crucial for DNA synthesis and also for cellular metabolism integrity. Additionally, it is vital for cell growth and maturation processes. In healthy cells FR-α receptor tends to be absent or barely detectable. Its significant expression has, on the other hand, been detected in ovarian, breast, renal and colon cancers. Overexpression of FR-α is also present in some primary CNS tumors, such as anaplastic ependymoma or papillary plexus carcinoma. A decreased expression of 35 genes including PRL, FSH, LH and TSH-β was detected in NF adenomas, as compared to other tissues. In PRL-secreting adenomas 17 genes were specifically overexpressed, whereas 30 were underexpressed. A significant overexpression of TG-β, trichohyalin, protease inhibitor 12 (PI-12) and PRL was found in these tumors. In contrast, FSH, LH, TS-β and GH genes were found to be underexpressed. A very low ODC gene expression was revealed. ODC gene encodes limiting enzyme in polyamines turnover. A low expression of ODC gene may be connected with dysregulation of the cell division process or differentiation of tumor cells. Other studies have found an overexpression of ODC gene to lead to neoplasia and tumor progression. The ODC overexpression has been detected in malignant gliomas, primitive neuroectodermal tumors (PNET), medulloblastomas, meningiomas, as well as in bladder, breast, prostate, esophageal and colon cancers, and other malignant neoplasms. In GH-secreting adenomas, 30 genes were specifically expressed with 15 significantly overexpressed, including ODC and GH genes, and the remaining 15 ones, including PRL, FSH, LH and TSH-6 were underexpressed. In ACTH-secreting adenomas, 51 genes were specifically expressed, with 19 significantly overexpressed, including GH receptor gene, TG-β and CMP-tk gene (gene of a transmembrane receptor containing intrinsic tyrosine kinase activity whose role is to control growth and cell differentiation processes). 32 genes were underexpressed, including PRL, FSH, LH and TSH-β [20]. The data obtained with microarrays were independently confirmed by analyzing 39 tissues with RT-PCR method [19,20].

In more recent studies, Galland *et al*. searched for the potential biologic correlated of increased NF invasiveness. 40 tissue specimens were examined, including 22 samples from patients with clinically aggressive disease and 18 non-invasive ones. They were analyzed with Agilent oligonucleotide microarrays containing 44 thousand probes. As a result, 346 genes differentiating invasive and non-invasive tumors were found. In invasive tumors 233 genes were overexpressed and 113 were underexpressed. The findings were confirmed with qRT-PCR test for which 35 genes were selected. The overexpression was confirmed only for *IGBP5, MYO5A, FLT3, NFE2L1*. In proteomic investigations only myosin 5a was overexpressed in invasive tumors [30]. Also Hussaini *et al*. investigated the genetic signature of increased invasiveness of pituitary adenomas. They analyzed 8 adenoma specimens, including 3 invasive and 5 non-invasive ones, with an Affymetrix microarray containing over 50 thousand gene probes, later confirming the findings in a RT-PCR test. They were able to obtain a group of genes differentiating subgroups of adenomas. It contained a thrombospondin gene, *MMP, MMP-9, MMP-14* [36].

The above findings seem particularly interesting in terms of a potential gene therapy or adjuvant therapies such as immunotherapy, radioimmunotherapy and chemotherapy. To give an example, an FR-α molecule is currently being examined in this respect in neoplasms like ovary or renal cancer. Potential therapies may involve the use of folate analogs of cytotoxic activity specific to cytoplasm cells. Anti FR-α antibodies alone or conjugated with a radioisotope could also be used to selectively target and kill NF adenoma cells. Folic acid analogs conjugated with a radioisotope would also act in a similar way. Molecules of FR-α, specific to NF adenoma, could as well be used to aid the imaging of adenomas with nuclear medicine or radiologic methods. It would be particularly useful in pituitary microadenoma diagnosis, recurrence detection, treatment monitoring and guiding the extent of resection. It seems possible to apply FR-α based diagnostic modalities for image-guided neurosurgery as well.

Another approach in the research on the molecular biology of pituitary tumors is extensively used, applying viral vectors to inhibit or augment the expression of desired genes. The use of adenoviral, retroviral, lentiviral, AAV or HSV vectors has been postulated [42], with constructs containing the specific promoters of pituitary hormones in the vector, and achieve that way an expression restricted to the pituitary cells. The therapeutic genes transferred into the cell may drive the production of specific enzymes, prodrugs, suppressor genes, toxins, growth inhibitors and other [42].

Factors selectively destroying an adenoma cell may be delivered also with non-viral vectors such as liposomes. Molecules specific to adenomas, like FR-α, could serve as targets for these agents. This way a wide range of active substances like strong toxins, enzymes and pro-enzymes, as well as growth and proliferation inhibitors can be delivered into the cell. Genes responsible for induction of apoptosis e.g. *p53, Rb, FAS, BAX, BCL-XS, HSV-TK,* and caspases can be a very good target for gene therapy. Gene therapy would also be helpful in pituitary function recovery after tumor resection. The use of vectors may result in supplying the cell with and/or multiplying gene copies responsible for the expression of the lacking hormone [41,42]. In spite of very actively conducted research, attempts to employ gene therapy in the treatment of pituitary adenomas are at present being carried out in vitro and in animals only [8,13,29,31,42].

Epigenetic abnormalities

A growing interest in epigenetic changes leading to tumor formation, recently driving a number of studies, has been stimulated by the discrepancies between an aberrant expression of specific proteins without apparent genetic alterations. It is already well-recognized that hypomethylation of CpG dinucleotides throughout the genome, especially aberrant methylation of CpG islands in gene promoters can play an important role in the formation of pituitary adenomas and their clinical behavior. The knowledge about the human methylome is, however, still incomplete, and only random data, mostly concerning promoter methylation of known oncogenes, are available. Methylation of *CDKN2A* (*p16*) or *RB* genes is a known phenomenon responsible for silencing of these genes in pituitary adenomas. *CDKN2A* promoter methylation is thought to be an early event in adenoma formation and was already described over ten years ago [64]. Similarly, silencing of *RB1* gene can be caused by promoter methylation in adenoma cells without *RB1* gene mutations and not expressing the pRB protein, while *RB1* gene inactivation by mutation is an unusual event [34,58]. Recently, it was found that another potent tumor suppressor gene *CDKN2C (p18(INK4c))* can also be involved in pituitary adenoma development. Frequent methylation of *CDKN2C* promoter was revealed in adenoma specimens lacking *CDKN2C* protein p18, also LOH of the *CDKN2C* locus was detected in 25% of cases, both correlating with the absence of p18 protein, while no mutations in coding regions of the gene could be demonstrated [39]. Expression of another tumor suppressor gene *RASSF1A* can also be regulated by epigenetic alterations. *RASSF1A* gene promoter methylation was found in human pituitary tumor samples and was most common in tumors showing most aggressive clinical behavior [55].

A more detailed analysis of the methylation pattern in pituitary tumors could shed some light on deregulation of genes potentially important for the formation of benign, well-differentiated adenomas. Aberrant methylation of *CDH13* and *CDH1* genes coding H-cadherin and E-cadherin, respectively, was observed. These are the proteins playing a crucial role in cellular adhesion and involved in cell motility/invasiveness. Methylation of *CDH13* and *CDH1* genes was found to correlate with their downregulation. This was then associated with more aggressive clinical behavior, since it was found mostly in invasive adenomas and grade IV

tumors [54]. *MEG3* gene hypermethylation was detected in non-functioning adenomas and it has been suggested that it may also be involved in pituitary tumorigenesis [69]. A *MEG3* derived cDNA isoform – MEG3a was found to be a potent cell growth inhibitor which supports the role of this gene in pituitary adenoma formation [68]. A significant association between promoter methylation and loss of DNA-damage inducible, growth inhibitory *GADD45*γ gene was also revealed in human pituitary adenomas, suggesting its contribution in hypophyseal tumors development [4]. Another gene which may be involved in pituitary tumorigenesis is pituitary tumor derived apoptosis gene (*PTAG*). Methylation of *PTAG* promoter was found in pituitary adenoma specimens using methylation-specific PCR technique in a study designed to find novel genes that are differentially methylated as compared to a normal pituitary gland [4,5].

Apart from hypermethylation and associated gene silencing, also aberrant hypomethylation may contribute to the development of pituitary tumors by transcriptional activation of otherwise inactive oncogenes or unmasking of repetitive elements, which can lead to genomic instability [25,33]. The mechanism of aberrant hypomethylation has recently been described as the cause of *MAGE-A3* gene activation in human adenoma cells. In normal pituitary the promoter of *MAGE-A3* gene was found to be extensively methylated, preventing the gene expression in normal hypophysis [71]. Furthermore, Zhu *et al*. showed that *MAGE-A3* can influence p53 regulation thus showing that the function of this anti-oncogene can be altered by mechanisms other than mutation. Mutation of *TP53* gene is commonly found in other types of neoplasms and in a subset of pituitary carcinomas, but is not present in pituitary adenomas [71,72]. Moreover, expression of *MAGE-A3* promoter was found in tumors lacking FGFR2-IIIb [71]. *FGFR2* gene downregulation was recently described in human pituitary adenoma cells, and its inactivation can also be attributed to epigenetic silencing. *FGFR2* gene promoter methylation was found in 45% of examined human adenoma samples [72]. It was suggested that FGFR2 downregulation can influence cell cycle progression by altering p27 expression and Rb phosphorylation [71,72].

There is growing evidence of epigenetic changes influencing cell cycle control and apoptosis, not always by altering expression of genes directly involved in these processes, but also by altering expression of other genes of regulatory function. The significance of their functions and reciprocal interactions of their products with proteins involved in vital cell activities is still mostly obscure and requires detailed investigation. Introduction of new, more exact and efficient methods of assessing a genome methylation pattern should allow for a comprehensive evaluation of epigenetic alterations associated with adenoma formation.

Conclusions

Molecular mechanisms responsible for the formation of pituitary adenomas, their biology, and, subsequently, their prognosis, still remain largely obscure. Significant biological and functional diversity of these benign tumors in spite of their monoclonality, poses a variety of issues still to be resolved. Hence, it is necessary to continue research into the molecular biology of adenomas, which would lead to a better understanding of their pathogenesis and biology, and to development of highly specific targeted therapies.

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