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MINI-SYMPOSIUM: Adamantinomatous Craniopharyngioma and Xanthomatous Lesions of the Sella

Proteomics in pediatric cystic craniopharyngioma

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Abstract

Adamantinomatous craniopharyngioma (ACP) is still often burdened by a poor prognosis in children as far as the risk of recurrence and the quality of life are concerned. Therefore, many efforts are now dedicated to investigate the molecular characteristics of this tumor aiming at finding new therapeutic options. ACP is prevalently a cystic lesion so that an increasing number of researches are focused on the analysis of its cystic content. In the present article, the main results of the current proteomic analysis (PA) on the ACP fluid are summarized. Both "bottom-up" and "top-down" approaches have been utilized. In the bottom-up approach, proteins and peptides are enzymatically or chemically digested prior to liquid chromatography and mass spectrometry analyses. The bottom-up approach pointed out several proteins of the inflammation (namely, α2-HS-glycoprotein, α1-antichymotrypsin and apolipoproteins) as possibly involved in the genesis and growth of the cystic component of ACP. The top-down strategy analyzes proteins and peptides in the intact state, making it particularly suitable for the identification of peptides and low molecular weight proteins and for the characterization of their possible isoforms and post-translational modifications. The top-down approach disclosed the presence of the thymosin β family. Thymosin β4, in particular, which is involved in the cytoskeleton organization and migration of several tumors, could play a role in the progression of ACP. Finally, PA was utilized to investigate alterations in cyst fluid character after treatment with interferon-α. The analyzed samples showed a progressive reduction of the levels of α-defensins (proteins involved in the inflammatory-mediated response) after the intracystic injection of interferon-α, thus reinforcing the hypothesis that inflammation contributes to ACP cyst pathogenesis. Additional studies on the solid component of ACP are still necessary to further validate the previous results and to identify possible markers for targeted therapy.

INTRODUCTION

Craniopharyngioma raises great interest in the pediatric research and clinical communities because of the characteristics that make the outcome of this tumor unfavorable for children. Adamantinomatous craniopharyngioma (ACP) often presents as large or huge mass, with invasion of neighboring eloquent or vital neurovascular structures, and high recurrence rate, even following gross total surgical resection. Because approximately 90% of ACP include a cystic component, multiple efforts have been directed toward assessing the effectiveness of intracystic therapies, such as intratumoral bleomycin (10, 33, 43), or to develop new ones, such as intracystic interferon-α (3, 4, 6, 7). Similarly, a new avenue in basic research on ACP is the characterization of cyst fluid (previous work summarized in Table 1), including the application of proteomic analysis (PA). Herein, we present a summary of the results obtained with PA at our institution. The goal of these studies was to assess the peptide and protein composition of the cyst fluid of this actively secreting tumor, in the hope of revealing critical aspects of the ACP tumorigenesis process, and potentially identifying molecular therapeutic targets.

METHODS

The analysis of proteins and peptides was undertaken through reverse phase liquid chromatography (RP-HPLC) in coupling with ESI-ion trap-MS (27) or high resolution ESI-LTQ-Orbitrap mass spectrometry (18). We then applied an integrated proteomic platform based on both a top-down and a bottom-up approach. Chromatographic separation of both undigested or digested protein acid-soluble fractions was performed in gradient elution using aqueous TFA (0.056%, v/v) solution, as eluent A, and water/acetonitrile mixture (20:80) 0.050% (v/v) TFA, as eluent

Table 1. Synopsis of the studies investigating the cyst fluid of craniopharyngioma (CP).

Authors and year	Materials and methods	Findings	Hypotheses
Schaub *et al* 1978 (31)	Measurement of β2-microglobulin concentration in CP and CSF of craniopharyngioma and gliomas	No correlation between CP and CSF β2-microglobulin levels in controls compared with tumors	Brain tumors can synthesize β2-microglobulin (in craniopharyngioma higher levels than low grade gliomas)
Harris *et al* 1988 (12)	Radioimmunoassay for β chain of β-HCG in CP and CSF	HCG material in all CP samples (9), in 5 tumors and in one CSF (after rupture of craniopharyngioma cyst) but not in serum	β-HCG is secreted in the CP independently of the serum levels
Szeifert *et al* 1991 (35)	CP histochemical and electrophoretic investigation	Mucin secretion in microcysts and electrophoretic pattern similar to normal serum	CP in craniopharyngioma is the result of a secretory action
Zumkeller *et al* 1991 (44)	IGF-I and II and IGF binding proteins search in CP	All these proteins are in the CP (IGF-II > IGF-I) several fold more than in the CSF	IGF-I and II and IGF binding proteins are secreted by craniopharyngioma
Honegger *et al* 1995 (13)	Measurement of HCG immunoactivity in intracranial cystic tumors	Elevated immunoactivity in CP of craniopharyngioma, moderate in pituitary adenoma and metastasis of lung cancer, absent in meningiomas and gliomas	HCG immunoactivity (β-HCG) can be used for differential diagnosis of brain cystic tumors: higher activity in craniopharyngioma
Zumkeller *et al* 1996 (45)	Search for IGF-I and II and IGF binding proteins in CP before and after treatment with 90-yttrium	IGF-II levels were higher in cyst fluid than CSF, while IGF-I levels were similar between cyst fluid and CSF. IGF-II levels decreased after intracavitary irradiation but with variable extent and duration	IGF-II (and IGF-I) and IGF binding proteins are secreted by craniopharyngioma and they can be involved in its growth
Arefyeva *et al* 2002 (2)	Search for markers of blood-barrier disruption in CP	Protein, albumin, lactate, IgG and IgM and PH were present in CF and correlated with their serum levels	Blood-barrier damage is the mechanism of cyst formation as it happens in other brain tumors
Mori *et al* 2004 (21)	Assessment of expression of IL-1α, IL-6 and TNF-α in CP	IL-6 much more expressed than IL-1α and TNF-α; IL-6 in CF > 50 000 times than CSF	IL-6 induces inflammatory reaction at the interface between craniopharyngioma and brain
Szeifert *et al* 2007 (34)	Histological, ultrastructural and electrophoretic changes in cystic craniopharyngioma after 90-yttrium treatment	Changes after treatment: epithelial layer destroyed, scarring tissue with hyaline degeneration, proliferative vasculopathy	Epithelium disruption and cyst fibrosis are the causes of cyst shrinkage and CP reduction after 90-yttrium treatment
Tena-Suck *et al* 2014 (37)	Injection of human CP on rat brain (intracortical)	Oxygen species formation, oxidative damage to lipids and reactive gliosis 15, 30 and 45 days after injection	CP causes inflammation, oxidative stress and cell damage in rat brain as it does in patients with craniopharyngioma

B. Sample pretreatment was optimized according to the proteomic approach employed following the procedures summarized in Figure 1. The two approaches are different but complementary to each other for the proteomic studies. Specifically, the top-down strategy, analyzing proteins and peptides in their intact state, is particularly suitable for the identification of peptides and low molecular weight proteins and for the characterization of their possible isoforms and post-translational modifications (PTMs). The bottom-up platform ("shotgun proteomics") is the most commonly used proteomic approach. Using this strategy, the proteins and peptides are enzymatically or chemically digested prior to HPLC-MS analysis. Therefore, large proteins can be successfully identified but incorrect identifications or loss of information on PTMs may occur.

RESULTS AND DISCUSSION

Overview

The ACP fluid of 19 children was analyzed. A first cohort of 6 patients (M/F: 2; mean age: 4.5 years) was enrolled to investigate changes in the cyst fluid protein composition after interferon-α administration. Following this pilot study, a second cohort of 13 patients (M/F: 1.15; mean age: 8.7 years) was studied to deepen the characterization of the fluid content through an integrated bottom-up and top-down proteomic platform.

Overall, the main identified proteins and peptides can be grouped within the following biological functions: inflammatory response (α-defensins, β-thymosins and apolipoprotein family), cell growth and differentiation (ubiquitin and β-thymosins), protein

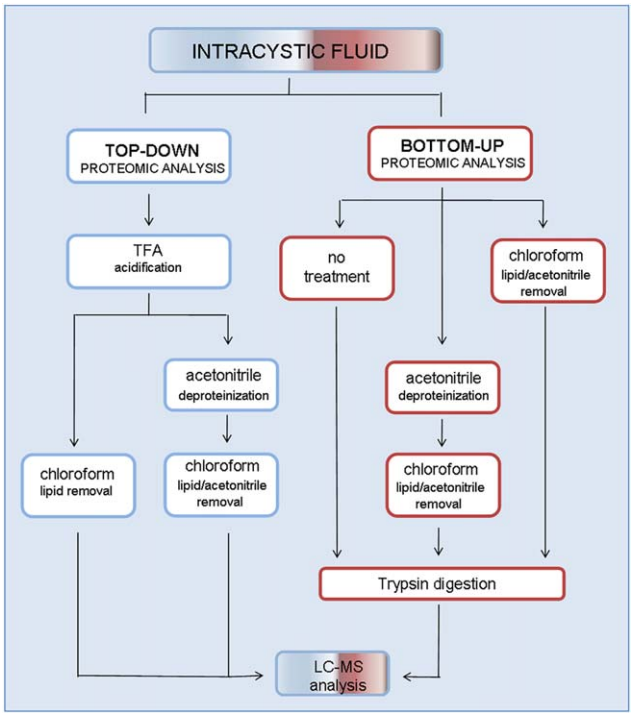

Figure 1. Proteomic integrated platform.

breakdown and degradation (cystatin C and ubiquitin), and lipid transport and removal (apolipoprotein family). A comparative analysis of the distribution of the proteins identified by top-down approach between intracranial cystic lesions of different histologiers, such as pilocytic astrocytoma (PiA) and Rathke's cleft cyst (RCC) (Table 2), 12 proteins were shared between these lesions and ACP (ubiquitin, apolipoprotein A-I, β-thymosins, des-TP apolipoprotein C-I proteoform, apolipoprotein A-II dimeric forms and cystatin C proteoforms, although the latter was most commonly detected in PiA). Ten proteins, most of which are associated with the inflammatory response, were identified exclusively in ACP and RCC (α-defensins, various hemoglobin fragments). Six proteins, mainly involved in the oxidative stress response, were primarily detected in PiA (apolipoprotein A-II and its cysteinylated form, apolipoprotein C-III, fibrinopeptide A and its C-terminal truncated form, fibrinopeptide B). The complement C3f fragment and β-microglobulin were also present specifically in PiA. Bottom-up proteomic analysis evidenced the apolipoprotein J exclusively in ACP. The gene ontology (GO) analysis of all proteins and peptides identified by the integrated top-down/bottom-up platform indicated their involvement in multicellular organismal biological processes (27%), binding (34%) and catalytic activities (33%) and transfer/carrier proteins protein class (50%) (Figure 2).

Bottom-up approach: proteins of inflammation

The following main proteins or groups of proteins were identified by this approach (18): (i) AHSG (α2-HS-glycoprotein or fetuin A), a serum glycosylated heterodimer whose secretion seems to be related to tissue mineralization and mineralization pathological processes. This protein is involved in the binding of small clusters of calcium and phosphate. To date, it has been correlated with the survival of patients with glioblastoma (normal/low serum levels of AHSG as predictor of longer survival) (28) and used as diagnostic serum biomarker in breast cancer (41). In ACP, its secretion in cyst fluid could potentially contribute to the presence of calcium flecks and inflammation, through its scavenger and anti-inflammatory properties; (ii) ACT (α1-antichymotrypsin), a secreted glycoprotein of the serpin family which acts as an inhibitor of chymotrypsin-like serine proteases and mast cell chymases. Its activation in the acute phase of inflammation could give reason of the fluid secretion in ACP. ACT has been found to be up-regulated in the CSF of patients with glioblastoma and in the serum of those with breast cancer (higher expression in the most malignant and metastatic histotypes) (19); (iii) Apolipoproteins (APO) C-I, A-I and J, a group of proteins involved in lipid transport and metabolism and considered to be the most relevant in ACP fluid. APO C-I is mainly synthesized in the liver, secreted in the plasma and expressed in the brain. It participates in the removal of cholesterol and tryglycerides from tissues. APO A-I is involved in inverse cholesterol transport and cholesterol esterification; and APO J, which is present as a glycosylated heterodimer in most mammalian tissues and fluids, has a similar action. APO C-I demonstrates altered expression in Alzheimer disease, and is a potential biomarker for various tumors (breast cancer, non-small cell lung cancer, pediatric nephroblastoma, etc.) (23, 25, 40, 42). APO A-I, which has anti-inflammatory and antioxidant properties, shows decreased expression in schizophrenia and seems to be independently regulated in the central nervous system (mismatch between serum and CNS levels) (14).

Top-down approach: β-thymosins

This approach demonstrated high levels of β-thymosins in ACP cyst fluid (9). First isolated from the calf thymus, β-thymosins are a family of 16 peptides (molecular weight about 5 kDa) including thymosin β4 (Tβ4, which is the most abundant in human cells), Tβ10 and Tβ15. Tβ4 and Tβ10 are also present in extracellular fluids, but it is still unknown if this results from cell damage or a secretory process (16). Tβ4 is the major G-actin-sequestering molecule in mammals where it regulates the organization of the cytoskeleton, thus influencing cell differentiation, migration and morphogenesis (8). In addition, it promotes angiogenesis, tissue repair and tumor growth (29). Expressed also in the central nervous system (in both children and adults), Tβ4 is involved in synaptogenesis, axon growth, plastic changes, and cell migration (8). Upregulation of β-thymosins has been associated with several diseases of the central nervous system (Alzheimer disease, brain ischemia, Huntington's disease, hippocampal denervation) (30, 36, 38). High levels are also detected in various tumors and tumor metastasis (Tβ4 can inhibit apoptosis and induce resistance to immune surveillance). As such, Tβ4 is consistently regarded as a potential therapeutic molecular target (39) or potential tumor marker (5). The role of thymosins in pathological conditions and tumor metastasis has been recently reviewed (32).

According to our results, β-thymosins were present in 10 out 13 (77%) samples of ACP cyst fluid (Tβ4 was 10 times more highly expressed than Tβ10). Interestingly, these peptides were not detected in the patients' CSF nor in control CSF (6 children with congenital hydrocephalus), thus suggesting their selective secretion in tumor cyst fluid. Their role in ACP could be related to the development and growth of the tumor cyst (binding G-actin monomers

Table 2. Comparative distribution of top-down approach detected proteins in tumor cyst fluids of PiA, AC and RCC.

Uniprot accession number	Protein name	PTMs	PA				AC													RCC	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
P68871	Hemoglobin subunit beta frag. 1-31								•		•			•		•	•		•		
P69905	Hemoglobin subunit alpha frag. 1-32							•	•	•	•			•		•			•	•	•
	Hemoglobin subunit alpha frag. 1-33						•				•			•		•			•	•	•
	Hemoglobin subunit alpha frag. 1-78						•	•						•			•		•	•	
P59665	Neutrophil defensin 1	3 S-S					•	•	•	•	•	•				•				•	•
	Neutrophil defensin 2	3 S-S					•	•	•	•	•	•				•				•	•
P59666	Neutrophil defensin 3	3 S-S					•	•	•		•	•		•		•			•	•	
P62328	Thymosin beta 4	Acetylation (N-term)	•	•	•	•	•	•		•	•	•	•	•		•		•	•	•	•
P63313	Thymosin beta 10	Acetylation (N-term)	•	•	•	•	•	•		•	•	•	•	•		•		•	•		
P0CG47	Ubiquitin		•	•	•	•	•	•	•	•	•			•	•	•	•	•	•	•	•
P02654	Apolipoprotein C-I	Des-TP (N-term)	•	•	•	•	•	•	•	•		•	•	•			•	•		•	•
	Apolipoprotein C-I		•	•	•	•	•	•													
P02647	Apolipoprotein A-I		•	•	•	•	•	•	•	•	•	•	•			•		•			
P02652	Apolipoprotein A-II	Homodimer 2 pyro-Q (N-term); 2 S-S	•	•	•	•	•	•	•	•		•	•				•	•	•	•	
	Apolipoprotein A-II	Apo A-II and des-Q (C-term) Apo A-II; 2 pyro-Q; 2 S-S	•	•	•	•	•	•	•	•		•	•				•	•	•	•	
	Apolipoprotein A-II	Homodimer 2 des-Q (C-term) Apo A-II; 2 pyro-Q (N-term); 2 S-S	•	•	•	•	•	•	•	•		•					•	•	•		
	Apolipoprotein A-II monomer	Pyro-Q (N-term)	•	•	•	•	•									•	•		•	•	•
	Apolipoprotein A-II monomer	Pyro-Q (N-term); cysteinilation C^{6}	•	•	•	•				•			•								
P01034	Cystatin C		•		•	•		•										•		•	
	Cystatin C	2 S-S; oxidation M^{14}	•		•	•	•		•	•								•			
	Cystatin C	2 S-S; des-SPPG (N-term)	•		•	•			•	•							•	•		•	•
P02671	Fibrinopeptide A		•	•	•	•															•
	Fibrinopeptide A	Des-A (C-term)	•	•	•	•		•								•					•
P02675	Fibrinopeptide B		•	•	•																
P01024	Complement C3 f frag.		•	•	•	•															
P02656	Apolipoprotein C-III	O-glycosyl (GalNAc, Hexose, Sialic Acid) T^{94}	•	•	•	•												•			•
P61769	Beta 2 microglobulin	1 S-S	•	•	•	•															

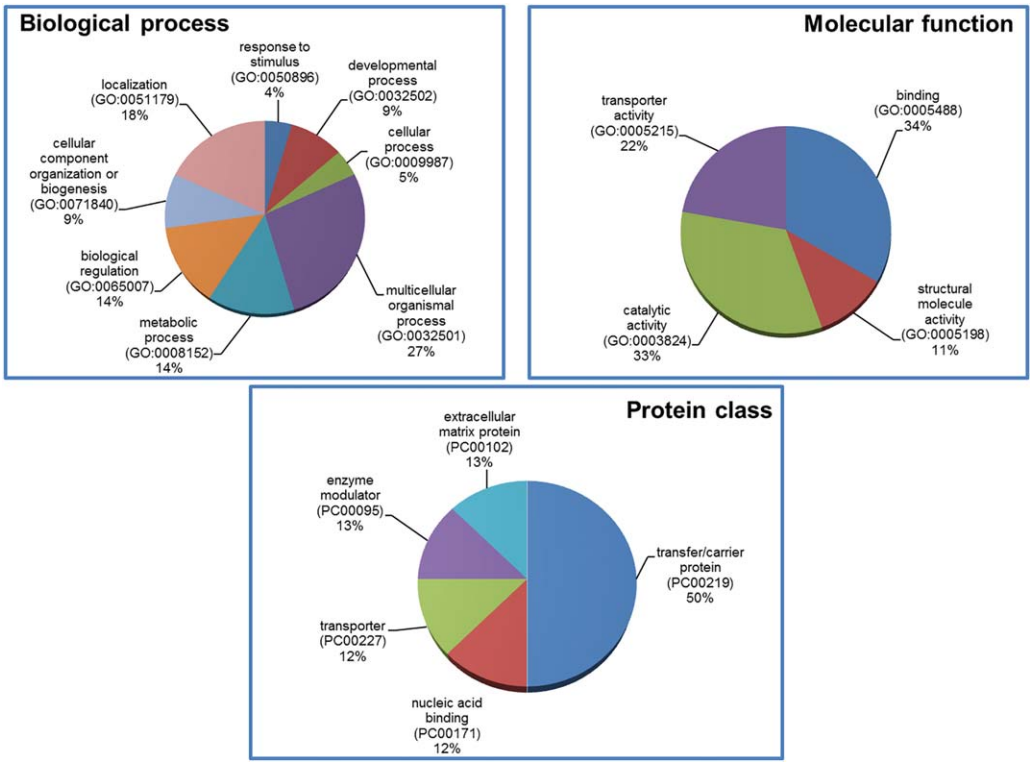

Figure 2. GO analysis of the proteins identified by the integrated top-down/bottom-up platform.

and inhibiting apoptosis). Existing literature (1, 22) indicates that Tβ4 upregulation (and potentially paracrine secretion) could promote ACP cell motility, progression and invasion through the activation of matrix metallopeptidase 7 (MMP-7) and c-Myc, following the stimulation of β-catenin signaling via down-regulation of E-cadherin. A recent description of β-thymosin peptides as potential markers of tumor aggression in posterior fossa brain tumors (17) supports the further investigation of these molecules in ACP solid tissue, which is now underway.

Proteomic analysis and interferon-α (IFN-α) treatment

A further family of peptides identified using the top-down approach is the neutrophil-defensins, specifically α-defensin 1, 2 and 3 (27). These were detected in each of our ACP fluid samples, showing a high expression (≥100 nanomoles) in more than a half of the cases. Interestingly, after the administration of IFN-α, a significant reduction of their levels was noted in the cyst fluid of treated patients (3 cases), the mean level dropping from 127.2 to 10.8 nanomoles. Each of these cases also demonstrated substantial shrinkage of the cystic ACP after INF-α treatment. α-defensins have antibacterial and antiviral activity, as they represent 30%–50% of the protein content of the azurophil granules of neutrophils (11). Increased levels of these peptides are found in the saliva of patients with oral squamous cell carcinoma, in the fluid of jaw cysts, and in the plasma of patients with sepsis (15, 20, 24). Their role in the inflammatory-mediated response to tumor is further supported by the high levels that we found in ACP patients before treatment, where their activity could stimulate the secretion of cyst fluid by the cyst wall epithelium. As a result, the decrease of α-defensins in the post-treatment period may account for the reduced fluid production and the cyst shrinkage. IFN-α may reduce α-defensin levels through an antitumoral effect on the squamous epithelial cells, an immuno-modulatory action on the recruitment of inflammatory cells, and anti-angiogenic activity (26). The high levels of α-defensins in ACP cystic fluid indicate no blood barrier disruption for the cyst formation since the serum levels of α-defensins is very low.

CONCLUSION

The proteomic characterization of ACP cyst fluid has led to the identification of proteins strongly connected with mineralization process, lipid transport, and the inflammatory response, which is in accordance with ACP cyst fluid composition (lipids, granules of cholesterol, flecks of calcium). These findings suggest a role for inflammation in the development of cystic ACP (cyst formation and growth). IFN-α modifies the composition of the cyst fluid by reducing α-defensins and may thereby induce cyst shrinkage through an anti-inflammatory mechanism. Moreover, some peptides (namely, β-thymosins) may be involved in ACP tumor growth, progression and infiltration. These merit further study to identify possible markers for targeted therapy.

The proteomic profile of ACP is similar to RCC, which may reflect a shared embryonic origin. Similarly, the ACP proteomic profile largely differs from that of other cystic tumor fluids (like PiA), thus supporting the unique origin of ACP cyst fluid.

Other than representing a useful approach for the molecular characterization and elucidation of possible mechanisms involved in ACP onset and progression, PA can lead to the individuation of biological markers of disease progression and recurrence; and molecular targets for therapy development. However, this technique has drawbacks. Cyst proteomics generate huge amounts of data, which may also demonstrate significant individual variability. The procedure of protein sequencing and characterization is also highly time consuming. Currently, advances in data elaboration software and the availability of sophisticated mass spectrometry instrumentation help overcome these barriers, facilitating the integration of proteomic information with the genomic and transcriptomic data. PA of the solid portion of ACP is now under study at our institution with the goal to complete the proteomic profile of this component of the tumor.

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