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**“Craniopharyngiomas: immunohistochemical
evaluation of prognostic factors.”**

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I. Introduction: clinical and pathological aspects of craniopharyngioma

1.1 Historical background

Craniopharyngiomas (CPs) are a heterogeneous group of benign but locally aggressive epithelial tumors arising within the sellar region, along the path of craniopharyngeal duct.

In 1847, Friedrich Zenker identified the presence of a proliferation of squamous cells at the tuberalis pars and pars distalis of pituitary. In the coming years a number of studies were carried out on adenohypophysis squamous epithelial cells. In 1899, the English pathologists Frederick Mott and Max Barrett, studying different epithelial tumors of the sellar region, were the first to hypothesize that these tumors originate from the residues of Rathke's pouch. This hypothesis was supported by Jakob Erdheim in 1904, after a systematic study of squamous epithelial cells of adenohypophysis. In the following years many terminologies were used until, in 1932, Harvey Cushing introduced the term craniopharyngioma unifying thus, under a joint name, cystic and solid tumors that originated from epithelial remnants attributed to the imperfect closure of the ductus craniopharyngeal (1, 2).

The CPs are benign neoplasms, histologically grade I sec WHO (2007), of which there are two subtypes: the adamantinomatous (adaCP), which is the most frequent and the papillary (papCP), rarer. The two subtypes differ in terms of frequency, age distribution, morphological and biological aspects and also show a different prognosis and clinical outcome. The adaCP, in particular, is a neoplasm often clinically aggressive and associated with a poor quality of life of patients (3).

1.2 Epidemiology

The CPs represent from 1.2 to 4.6% of all intracranial tumors and are the most common not neuroepithelial malignancy in childhood, constituting, in this age group, 5-10% of all intracranial tumors and the most frequent pituitary tumor. These tumors are more common in Nigerians (18% of all cancers of the central nervous system) and Japanese children (annual incidence of 5.25 cases for million people in the pediatric population). Not observed gender predilection (3). As regards the age of onset, it recognizes a bimodal distribution in adamantinomatous subtype, with a first peak in children between 5 and 15 years and a second peak in adults between 45 and 60 years (3-5). The papillary subtype occurs almost exclusively in adults, with an average age of 40-55 years. The CP may originate in any of the product segment craniopharyngeal, from the sella turcica to hypothalamus, however, the most frequent location (95%) is suprasellar region, with a minor component intrasellar (3, 6, 7). They have been described rare primary sites, such as at the level of sphenoid sinus and nasopharynx (1, 8, 9), which have their origin, likely, by ectopic pituitary tissue pharyngeal and which must be distinguished from secondary neoplastic invasion, characteristic of these tumors .

1.3 Clinical, biochemical and radiological aspects

Symptoms are non-specific and are related to the effect of the tumor mass in sellar region. The majority of patients present with headache (75%) and visual disturbances (62-84%), the latter, more frequent in adults, including diplopia, papilledema, and various patterns of vision loss (3).

Other symptoms include behavioral disorders (irritability, memory loss, learning delays in children), nausea, vomiting, obesity, drowsiness and symptoms of pituitary hormone deficiency, the latter observed in 52-87% of patients, particularly in children (3, 10).

Endocrine disorders can be caused or by destruction of pituitary tissue or interruption of the pituitary stalk and loss of hypothalamic feedback. The hormonal deficiency include those for GH (75%), LH / FSH (40%), ACTH (25%) and TSH (25%) (3). In children the most common endocrine disorder is represented by the growth defects (up to dwarfism), less common premature or delayed puberty; in adults, however, are frequent sexual dysfunctions: impotence primary in men or secondary amenorrhea in women. Diabetes insipidus is present in more than 17% of pediatric patients and in more than 30% of those adults (1). Finally intracranial hypertension-related signs may be present, especially in cases with compression or invasion of the third ventricle (3, 10).

The onset of symptoms is variable, in relation to the speed of tumor growth: some patients may present an acute clinical picture, characterized by rapidly progressive symptoms such as morning headaches, vomiting and double vision; others may manifest a subacute onset and slower clinical course.

Laboratory tests show a variable level of hypopituitarism, with reduction of baseline hormone levels or with normal hormone levels but a reduced response to stimulation. Patients with hypopituitarism may have reduced levels of thyroid and glucocorticoid hormones. In some patients there is a mild hyperprolactinemia caused by the interruption of the pituitary stalk and loss of hypothalamic control (1). It's possible an association of CP with a pituitary adenoma. In

case of prolactinoma it will present a more marked hyperprolactinemia (11, 12) while in case of adenoma thyroid stimulating hormone may be occur thyrotoxicosis (13). They are described cases of CP associated with lymphocytic hypophysitis resulting in complete failure pituitary (14).

The most frequent neuroimaging findings in patients with CP (50% of cases) are expansion or erosion of the sellar region and calcifications of suprasellar region. Computed tomography (CT) and magnetic resonance imaging (MRI) are the most widely used as diagnostic methods. The TC, in fact, is able to identify calcifications with greater precision; it assesses tumoral structure, generally cystic or solid-cystic, more rarely only solid; it defines the site of the tumor (suprasellar in 75% of cases; suprasellar with intrasellar component in 21% of cases; fully intrasellar in 4% of cases) and tumoral extension. MRI allows to define the extent of the particular tumor, and, relationships with surrounding structures; it differentiates with greater accuracy the solid component (isointense and heterogeneous signal) from the cystic (hyperintense signal in the absence of contrast medium in T1 weighted sequences) component; it evaluates the possible involvement of the 3rd ventricle and the optic chiasm (15-17).

1.4 Pathological anatomy

1.4.1 Macroscopy

Most CPs appear as a suprasellar mass of variable dimensions, usually more than 1 cm at the time of diagnosis. The adamantinomatous subtype shows a lobulated or solid or spongy aspect based on the presence of a variable cystic component. In section, the cysts often contain characteristically a dark brownish liquid. The macroscopic appearance reflects, also, the presence

of secondary retrogressive change, such as fibrosis, calcification, ossification and presence of cholesterol deposits. The neoplasm does not present a capsule interface with the brain parenchyma and often infiltrating the surrounding brain parenchyma. The papillary subtype is a well-circumscribed tumor, solid or rarely cystic; it does not show neither calcification nor accumulations of brownish.

1.4.2 Histopathology

The CPs are intracranial tumors of neuroepithelial nature divided in two subtypes: the adamantinomatous (adaCP) and papillary (papCP) type. The adaCP constitutes the most frequent subtype of CP. This neoplasm recalls the dental enamel organ and, therefore, histologically is similar to adamantinoma. Microscopically the tumor consists of nests, cords and irregular trabeculae of squamous epithelial cells grouped into lobules with multinodular configuration, Peripherally, a columnar epithelium with available "palisade" is present; in the central areas, the cells are arranged in a fibrous stroma and appear interconnected by thin extensions, assuming a starry myxoid aspect (reticulum starry). Cystic spaces lined with flattened epithelium, filled with amorphous material and nodules of "wet keratin" are present. Another peculiar morphological appearance of adaCP is the presence of clusters of neoplastic cells with morphology "whirl-like"; such clusters are observed in varying amounts in almost all cancers and are often localized on the tumor invasion front. It may be present, in addition, regressive phenomena such as dystrophic calcification, footprints of cholesterol crystals, often associated with a chronic inflammatory infiltrate of granulomatous type, with multinucleated giant cells and macrophages engulfing haemosiderin pigment. At the interface with the brain parenchyma it is frequently present reactive

gliosis, with abundant fibers of Rosenthal (1, 3) (**figures 1-2**). The papCP is rarer than adaCP. Histologically it is characterized by a monomorphic proliferation of well differentiated squamous epithelial cells without maturation surface, often arranged in buds or lax pseudopapille around a fibrovascular core, sometimes with a chronic inflammatory infiltrate of varying amounts. Unlike the adaCP, are not observed images of "wet keratin", footprints of cholesterol crystals nor calcifications (1, 3) (**figures 3-4**).

1.4.3 Ancillary techniques

The histological features of these tumors are quite characteristic and, therefore, ancillary methods are not required for the purposes of diagnosis. However, CPs are characterized by the expression of low and high molecular weight cytokeratins (1). In particular, the CPs show positivity for cytokeratin 7 and cytokeratin 8 and negativity for cytokeratin 20; this immunophenotypic profile is useful for the differential diagnosis of CP with Rathke cleft cysts.

Electron microscopy shows typical ultrastructural features of keratinizing epithelial cells, namely the presence of numerous tonofilamenti, solid intercellular junctions and the absence of secretory granules (1, 3, 18).

1.5 Differential diagnosis

The CPs with extensive cystic component can create problems of differential diagnosis with other cystic lesions typical of the sellar region, in particular with epidermoid cysts and those of Rathke's

pouch. Epidermoid cysts are unilocular, bordered by a stratified squamous epithelium of uniform thickness and rarely calcified. Rathke cleft cysts instead are bordered by a ciliated cubic or columnar epithelium with occasional "goblet cells", and focal areas of squamous metaplasia. In these cases, immunohistochemistry can aid in the differential diagnosis since the CP does not express cytokeratin 8 nor cytokeratin 20, while both the cysts Rathke cleft that the normal cells of the pars intermedia of the pituitary express both markers (1, 19).

It must be remembered, finally, as the infiltrative nature of CP often causes a reactive gliosis in the surrounding brain parenchyma, where it is particularly exuberant, it should not be confused with a pilocytic astrocytoma.

1.6 Therapy

The complete surgical excision of adaCP is the first choice of treatment, however, it is quite difficult to practice, since it is associated with a high morbidity rate caused by endocrine dysfunction and severe neurological deficits (20). For these reasons a less aggressive surgical method is preferred, followed by radiotherapy on residual tumor (21). The rate of postoperative recurrence is high and amounts to 23% and 27% respectively as a result of total resection or subtotal resection associated with adjuvant radiotherapy, in a post-operative period of five years (22, 23).

Currently, there is no consensus and there are no guidelines for the treatment of CPs. Surgical removal of the CPs is performed through different approaches microsurgical. The choice of approach depends substantially on the site of the lesion (24, 25). The evolution of surgical

techniques has led, in recent decades, to a net reduction in mortality and an increase in the effectiveness of the intervention. The early mortality is, in fact, equal to 2.6% after transphenoidal surgery and to 3.1% after transcranial surgery (26). In recurrent tumors the total removal could be obtained in a percentage of cases lower than in primary tumors (0-25%) and is associated with increased mortality and perioperative morbidity (10.5-24%) (27).

For cases in which no total removal is practicable (for example neoplasms of dimensions greater than 4-5 cm or recurrent tumors), the standard approach consists in subtotal removal followed by a traditional adjuvant radiotherapy (28).

The stereotactic radiotherapy, both in the form of radiosurgery (RS) that of fractionated stereotactic radiotherapy (FSrt), constitutes a more accurate irradiation technique that allows a more precise localization of the tumor with consequent reduction of the normal brain parenchyma volume exposed to high doses of radiation therapy (29-31).

Currently there is no effective drug therapy for CP; the only treatment is surgery and / or radiotherapy.

1.7 Prognosis

More than 85% of patients with CP survives after three years of diagnosis; mortality appears therefore usually contained, when the tumor is treated in a appropriate time. The overall survival rates, mainly described in pediatric series, reflecting the effects of various treatments and vary from 83 to 96% at 5 years and 65 to 100% at 10 years, with an average of 62% at 20 years (2).

In adult or mixed population (adult-children) the survival rate is 54-96% at 5 years and 40 to 93%

at 10 years (2), with a recurrence-free survival of 60-93% at 10 years (3). The lower limits of survival rates depend on data derived from older studies, ie before modern advances in neurosurgical techniques, neuroimaging and radiotherapy.

The late mortality can be caused by the progressive disease with multiple recurrences, chronic hypothalamic failure, hormone deficiency, cardiovascular diseases, convulsions (2).

The CP frequently involve a poor quality of life of affected patients, and because of the many long-term sequelae associated with treatments (surgical and / or radiotherapy), are often considered by clinicians as a chronic disease.

Finally, the risk of secondary cancer, as result of radiotherapy, is very low, with only a few cases reported in the literature, including two cases of glioblastoma, a case of glioma (grade malignancy unspecified) and a case of meningioma of the posterior cranial fossa (28, 32). The CP malignant transformation is very rare and is characterized by metastatic dissemination via leptomeningeal (33-35). The malignant component may show morphological features of a squamous cell carcinoma, myoepithelial carcinoma or odontogenic ghost cell carcinoma (1).

At present there is still no consensus about the factors that may influence the biological behavior of CP. The only established prognostic factor is the size of the tumor, with the highest survival rates found in the size of tumors less than 3 cm (2, 36), because, as already mentioned, the appearance of recurrence is closely related to the radicality of surgery, not practicable for the lesions of large dimensions, with a very poor prognosis for tumors larger than 5 cm (3).

As regards the histopathological aspects, the papillary subtype is usually associated with a lower tendency to recurrence and to a better prognosis than the adamantinomatous subtype or to mixed

forms (2). Furthermore, a morphological aspect significantly associated with tumor recurrence, is constituted by the presence of clusters of neoplastic cells with morphology "whirl-like", often present on the tumor invasion front. Even a marked reactive gliosis in the brain parenchyma perilesional, according to some authors (36, 37) is associated with relapses.

The proliferation index (Ki-67 / MIB-1) appears to be associated with relapses, however, it is extremely variable from case to case and has not yet been established a cut-off value (3); some authors have described a case of CP clinically very aggressive and characterized by rapid onset of relapse, with values of 20% and 15% respectively in the primary tumor and in the relapsed case (36).

1.8 Pathogenesis of CP

1.8.1 Pathogenetic theories

The CP is an intracranial tumor derived from the transformation of embryonic tissue. There are several hypotheses about what is the tissue of origin: residues ectodermal of Rathke cleft or embryonic epithelial residues of adenohypophysis.

The exact etiopathogenesis of these tumors is still unclear and highly controversial, however, several genetic and molecular aspects seem to indicate a distinct pathogenesis in two subtypes.

There are, therefore, two main etiopathogenetic theories: embryonal and metaplastic (2):

- Embryonic theory: according to this theory the adaCP derives from neoplastic transformation of squamous nests of embryonic cells left over by involution of craniopharyngeal duct. During the process of proliferation and rotation of Rathke's pouch cells leading to the formation of the

adenohypophysis, the residual cells of craniopharyngeal duct are scattered in the saddle and suprasellar region that are, in fact, the most frequent sites of CP's onset (2, 38-40).

- Metaplastic theory: according to this theory the papCP would result from metaplasia of pituitary cells of pars tuberalis resulting in the formation of squamous cell nests (2). This hypothesis is supported by the finding of metaplastic squamous nest in the pituitary gland (the number of which increases with age) and the presence of hormones in these nests. It been described a rare case of papCP whose features suggested a possible origin from the processing of residues of a cyst of Rathke's pouch (38).

Therefore, according to these theories, while the papCP would originate from the metaplastic transformation of mature epithelial cells of the anterior pituitary, the adaCP derives from less differentiated cells of Rathke's pouch, left over along the course of craniopharyngeal duct. In this process plays a fundamental role the Wnt pathway / beta-catenin (canonical WNT pathway) (23).

1.8.2 Wnt \ beta-catenin pathway

The pathway Wnt / beta-catenin is one of the main regulators of multiple cellular processes both in the embryo and in adults. This pathway, at the pituitary level, is involved in normal morphogenesis and tissue differentiation and, if activated abnormally, in cancerogenesis processes (41-43).

The Wnt proteins (Wingless Type) family consists of twenty members that act by binding to Frizzled receptors (Fz) which present a extracellular domain N-terminal rich in cysteine that links

the Wnt protein, seven transmembrane helices and a short cytoplasmic tail carboxy-terminal . For the activation of the system it is also required the presence of the co-receptor LRP (LDL-related protein) and the formation of the ternary complex (Wnt / Fz / LRP) (44).

The activity of the Wnt pathway / beta-catenin is controlled by the stability of beta-catenin, a transcriptional activator missing a binding domain with the DNA. In the absence of Wnt ligands, the beta-catenin is phosphorylated at specific amino acids residues encoded by exon 3 of CTNNB1 gene as part of a destruction complex consisting axina, complex APC (Adenomatous Polyposis Coli) and GSK-3 β (Glycogen synthase Kinase-3 β) with a proteasome degradation through pathway of ubiquitination. This prevents its stabilization and, consequently, the activation of the Wnt pathway. The binding of Wnt proteins to their receptors Frizzled (FZL) and co-receptor LRP cause the degradation of the destruction complex through the activation of Dishevelled (DVL), cytoplasmic protein scaffold, thereby preventing the phosphorylation of beta-catenin and consequently, its degradation. The stabilization of beta-catenin causes an increase of the cytoplasmic levels of the protein and its translocation to the nucleus where it can interact with the DNA binding transcription factors belonging to the TCF \ LEF family, thereby activating the expression of the target genes of the pathway (45). Some of the activated genes are important for cell cycle progression, such as c-myc and cyclin D1, others are inhibitors of apoptosis, such as survivin, or-promoting genes tumor progression, such as laminin- γ 2, and MMP7 (matrix metalloproteinase-7), that encode for proteins involved in the degradation of the basement membrane and extracellular matrix.

When the Wnt system is not stimulated, the transcription factors TCF / LEF remain associated with transcriptional co-repressors Gro (Groucho) and CtBP (C-Terminal Binding Protein), forming an inactive complex (46).

CTNNB1 mutations of the gene encoding the beta-catenin in exon 3 involve deletions or substitutions of amino acid residues critical for its degradation by the destruction of the complex causing, therefore, an increase in cytoplasmic levels of the protein, its expression aberrant nuclear and, consequently, a constitutive activation of the Wnt pathway (40-45).

Studies in mice genetically modified to obtain a mutated form of beta-catenin (transcriptionally active, but devoid of the amino acid residue encoded by exon 3 of CTNNB1 gene) have shown that the Wnt pathway /beta-catenin is required for the terminal differentiation of the pituitary cells in the last stages of development and should be inhibited in the early stages to allow the normal proliferation of the progenitors of Rathke's pouch. Un upregulation of the Wnt pathway / beta-catenin in progenitor Rathke cleft causes, in fact, pituitary hyperplasia, ectopic pituitary tissue in the oropharyngeal cavity and perinatal death in 80% of mice; the remaining 20% survive (an average of 11 weeks after birth) but with serious defects of growth and pituitary tumors showing histopathological aspects similar to those of adaCP and nuclear expression of beta-catenin and Axina2, LEF1 and BMP4, indicating the activation of the Wnt pathway (41, 47-50).

Although murine tumors differ from human ones for some aspects (absence of wet keratin or calcifications; faster postnatal onset), suggesting the existence of species-specific differences, mutations of the human beta-catenin are functionally equivalent to those of the models murine.

Mutations of the gene CTNNB1 therefore constitute the molecular hallmark of adaCP. These mutations were observed in the majority of human adaCP (about 70% of cases), predominantly affecting the exon 3 of the gene and, as already explained above, lead to the loss or replacement of critical amino acid residues for the stabilization of the protein thus blocking its degradation, nucleus-cytoplasmic accumulation (ie aberrant nuclear expression) and activation of the Wnt pathway (51-53). However, nuclear accumulation of β -catenin is observed in more than 94% of adaCP, suggesting that additional mechanisms, not yet known, probably of epigenetic nature, may be involved in the stabilization of the protein (23). Furthermore, although the CTNNB1 mutation is ubiquitous, the nuclear expression of β -catenin is observed only in small clusters of neoplastic cells; these clusters often show a "whirl-like" morphology and are exclusively observed in adaCP, never in papCP or other tumors of the sellar region (54, 55). Neoplastic cells of the cluster do not express markers of proliferation (Ki-67), pituitary hormones and are negative for synaptophysin.

1.8.3 Role of stem cells in the pathogenesis of CP

The presence of tissue specific stem cells has been well documented in many organs and tissues including, brain, gastro-intestinal tract, skin, bone marrow, and skeletal muscle. These cells are characterized by self-regeneration capacity ("self renewal"), by the expression of stem cell markers (such as SOX2) and can give rise to a progeny of cells able to differentiate and to contribute both to homeostasis, both to tissue regeneration (41, 56, 57). The presence of stem cells in the pituitary, although long hypothesized, was only recently demonstrated in murine models

and in humans. The pituitary stem cells express the transcription factors SOX9 and SOX2 (the latter only in the mouse), can differentiate into all pituitary cell lines hormone-producing and are crucial for homeostasis and regeneration of the gland (58-59). Tissue-specific stem cells can give rise to tumors, a concept that has given rise to the "hierarchical model" of cancer stem cell (CSC) (60). The term CST defines a neoplastic cell capable of self-regenerate, that is to give rise to another cell identical to itself (with tumorigenic capacity) and to generate a more differentiated neoplastic cell population (devoid of tumorigenic capacity). Therefore tumor is present in a minority population of CST responsible of tumor growth, maintenance of the compartment neoplastic stem and its progeny, non-tumorigenic, which constitutes the bulk of the tumor but is lacking in those characteristics of the CST. It is apparent how the staminal model considers the tumor not as a homogeneous population of cells rather as a heterogeneous proliferation characterized by a sort of "hierarchy" where the CST, though few, are the basic component of the development and maintenance of the neoplasm.

Another feature of the CSC is constituted by the resistance to chemotherapy and radiotherapy; this aspect may be responsible for tumor progression and relapse (61, 62).

The CSC may, therefore, represent potential targets for new therapies in oncology.

Recent studies, both mouse models and in human tumors, have investigated the presence of CSC in pituitary tumors, especially in adaCP (23, 49, 54, 58). The results of these studies suggest that the adaCP presented a minority population of cells with stem cell phenotype. These clusters are

characterized by the expression of aberrant nuclear beta-catenin and do not express the Ki-67, so they are in a non-proliferating status.

1.8.4 Molecular alterations of papillary craniopharyngioma

The molecular markers of papCP consists of the BRAF V600E mutation, found in a higher percentage of cases (95%), with a specificity of 100%, with activation of the MAP kinase pathway.

The CTNNB1 mutations and BRAF V600E in the two subtypes of CP are both clonal and mutually exclusive. These findings may have important implications in the diagnosis and treatment of these malignancies (63).

1.8.5 Role of ataxia-telangiectasia-mutated (ATM) protein Kinase

ATM is a known tumour suppressor which is frequently mutated in a broad range of human cancers including lung, colorectal, breast and haematopoietic cancers. ATM is a kinase activated by autophosphorylation (p-ATM) upon DNA double-strand breaks arising from errors during replication, byproducts of metabolism, chemotherapy or ionizing radiations. The initial scientific interest in ATM was, however, not focused on its role in tumour development. ATM was first described in 1995 as the gene defective in the autosomal recessive human hereditary disorder ataxia-telangiectasia (A-T). This disease, caused by a loss of ATM function, is characterized by progressive cerebellar degeneration, telangiectasia, immunodeficiency, genomic instability

cancer susceptibility and profound sensitivity to ionising radiation (IR); this hypersensitivity of ATM-defective cells to IR represents the main interest for ATM as therapeutic target for cancer therapy (64-65).

II. Scope of Work

Based on the above, the objectives of our work were as follows:

- To deepen the study of the pathobiology of the CP, in particular the role of β -catenin and stem cell;
- To study possible factors associated with the tendency to relapse, specifically in adamantinomatous subtype;
- To identify the prognostic and predictive markers that can be used by immunohistochemistry method.

To achieve these objectives we have therefore decided to:

- Evaluate, using immunohistochemical method, on a selected group of CPs:
 - 1) the expression of β -catenin;
 - 2) the proliferation index (Ki-67);
 - 3) the expression of stem cell markers: the CD166 (ALCAM), closely related to many types of tumors, with different prognostic outcomes (64); CD133 (Prominin 1), whose expression is associated with a poor response to radiotherapy (23);
 - 4) the expression of pATM
- To correlate the results obtained with the clinical aspects of neoplasms, or the presence or absence of tumor recurrence.

III. Materials and methods

3.1 Case Study

45 samples of craniopharyngiomas were analyzed (**table 1**). They were all obtained as formalin fixed tissue from the archive of the Department of Anatomic Pathology of the Federico II University Hospital of Naples. Patients underwent surgery at the Department of Neurosurgery of the same hospital. The cases were identified between 1998 and 2015 and the diagnoses were made in accordance with the World Health Organization (WHO) classification. Only specimens containing sufficient amounts of the respective CP subtype were taken into account.

For each patient follow-up data were available. Written informed consent was obtained from all patients.

Of 45 patients 30 were males and 15 were females. Age ranged from 2 to 77 years. Patients had been treated by complete surgical removal in 23 cases or by subtotal surgery followed by radiotherapy in 22 cases. In 38 cases the diagnosis was of adamantinomatous craniopharyngioma (adaCP) and in 7 cases it was of papillary subtype (papCP). Of 4 recurrent cases only the primary tumor tissue was included for statistical evaluation; in 9 cases only the tissue of the recurrence was available (8 adaCP and 1 papCP). Therefore 13 cases were considered recurrent and 32 not recurrent.

3.2 Immunohistochemistry

4 μ m sections were used for immunohistochemistry. Sections were dewaxed in xylene, hydrated in graded series of alcohol and subjected to heat-induced antigen retrieval. After blocking

endogenous peroxidase activity, the tissue was incubated with monoclonal antibodies for anti β -catenin (clone 14, mouse; Roche Ventana, 1:100 dilution), CD166 (clone MOG/07, ab49496, mouse; Abcam, 1:100 dilution), p-ATM (phosphoS1981)(clone ab81292;abcam; 1: 200 dilution) and Ki67 (Clone MIB1, mouse; Dako, 1:100 dilution) and with the polyclonal antibody CD133 (rabbit; Abcam, 1:100 dilution), all for 90 minutes. Subsequently, the slices were rinsed and incubated with the biotinylated secondary antibody, at room temperature, for 30 minutes. The bound antibody complexes were stained for 3-5 minutes or until appropriate for microscopic examination with diaminobenzidine, and they were then counterstained with hematoxylin (30s) and mounted. Appropriate positive controls were chosen: mammary fibromatosis for β -catenin, normal skin for CD166 and CD133 and normal pancreas for p-ATM. Negative control was obtained by omitting the primary antibody.

3.3 Scoring

All the slices were reviewed using light microscopy.

β -catenin was considered as positive only in case of nuclear staining and a total immunostaining score (IS) was calculated as the product of a Proportion score (**0**=0%, **1**<10 %, **2**=10-50%, **3**=51-80%, **4**>80%) and an Intensity score (**0**= no signal, **1**= weak signal, **2**= moderate, **3**= strong). The same IS was used also for CD133 and CD166 membrane signal. The 3 markers were then divided into two scoring groups, on the base of the IS: absent/low expression (IS=0-2) and moderate/high expression (IS=3-12).

For pATM, a different scoring system was adopted because no differences in intensity were noticed. It was based on the proportion of neoplastic cells showing a signal and on its cellular localization (C, cytoplasmic or N, nuclear): **0**=absent, **1**=<10%, **2**=10-30%, **3**>30%.

For the evaluation of the proliferating index Ki67 (Labeling Index, L.I.), "hot spot" areas were chosen and an average of the values on 5 adjacent fields (at least 500 neoplastic cells) was calculated: normally highly proliferating areas were excluded, namely basal cells. A cut-off of 5% was identified because the median value was of 6%.

In some cases not all the markers could be tested because of tissue exhaustion.

3.4 Statistical evaluation

The study of association between the scoring group of each marker (absent/low or moderate/high expression for β -catenin, CD133 and CD166 and $\leq 5\%$ or $> 5\%$ for Ki67) and clinico-pathological features (age, gender, recurrence and histotype) was carried out by Fisher's exact test. For pATM the population was divided into positive and negative cases.

Spearman correlation test was used to examine the correlation between β -catenin and, respectively, CD133, CD166 and Ki67 and between CD166 and Ki67.

A p value ≤ 0.05 was considered statistically significant. All tests were two sided and carried out with GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

IV Results

4.1 Immunohistochemical expression of β -catenin

β -catenin immunoreactivity was detected at the cell membrane in most cases and in the nucleus in 28 cases. We focused on the nuclear signal that was located in neoplastic cells forming "whirl-like" structures and in palisading basal cells arranged around the stellate reticulum. The signal was variable in intensity and proportion of distribution.

β -catenin immunostaining score was negative in 17 cases and in positive cases it ranged from 1 to 9, being 1, 2, 3, 4, 6 and 9 in 4, 11, 2, 7, 3 and 1 cases, respectively. Based on the calculated IS, all specimens were subsequently divided into two different scoring groups: 32 were absent/low (IS=0-2) and 13 were moderate/high (IS>2) (**figure 5-6**).

Out of 32 cases with absent/low β -catenin IS, 5 were recurrent while out of 13 with moderate/high β -catenin IS 8 were recurrent (**table 1**).

4.2 Immunohistochemical expression of Ki67

Ki67 immunostaining was available in 41 cases and it showed nuclear signal with a range of values between 1 and 25 %, a mean value of 8% and a median of 6% (**table 1**).

4.3 Immunohistochemical expression of CD166

CD166 was evaluated on 31 cases. The IS was negative in 7 cases and positive in 24 cases, ranging from 1 to 6 and being respectively 1, 2, 3, 4 and 6 in 5, 5, 4, 8 and 2 cases. 17 belonged to the absent/low (IS=0-2) scoring group while 14 to the moderate/high (IS>2) (**figure 7-8**).

The signal was mainly located at the cell membrane with diffuse distribution but sparing the whirl-like cell clusters that showed nuclear β -catenin accumulations in adaCPs. In papCPs the immunostaining was more homogeneous.

Out of 13 recurrent cases 3 showed a CD166-IS \leq 2, while in 10 cases the immunoscore was $>$ 2 (**table 1**).

4.4 Immunohistochemical expression of CD133

CD133 displayed membrane immunostaining with variable distribution in both adaCPs and papCPs and it did not localize exclusively in cell clusters, as already described in literature (**table 1**) (**figure 9**).

4.5 Immunohistochemical expression of pATM

27 cases out of 45 showed nuclear expression of pATM: 9, 11 and 7 cases with 1, 2 and 3 IS respectively. All papCPs showed nuclear reactivity (7/7).

In 23 out of 45 cases a cytoplasmic reactivity was detected (9, 10 and 4 cases showed 1, 2 and 3 IS respectively). Only 1/7 papCP and 16/28 adaCPs were positive (**table 1**).

4.6 Statistic analysis

The Fisher exact test showed a statistically significant correlation between the onset of relapsed tumor and:

- A moderate\high level of immunoreactivity of the beta-catenin ($p = 0.0039$);

- A moderate\high level of immunoreactivity of CD166 ($p = 0.0040$);
- High proliferation index (Ki-67) ($p = 0.0385$).

We have not found a statistically significant correlation between the expression of CD133 and the presence of recurrence ($p = 0.5962$) (**table 2a**).

With the Spearman correlation test, we found a statistically significant correlation between the expression of:

- β -catenin and Ki-67 ($p=0.0011$, $r=0.4903$);
- β -catenin and CD166 ($p=0.0002$, $r=0.6218$).
- CD66 and Ki67 ($p=0.0054$, $r=0.5119$);

We have not found a statistically significant correlation between β -catenin and CD133 ($p=0.4156$, $r=0.1543$).

We compared both pATM expressions, nuclear and cytoplasmic, in both CP subtypes, by Fisher's exact test and found out that NpATM was significantly more expressed in papCPs ($p=0.0313$), while CpATM was more present in adaCPs($p=0.0470$). No differences were found out when we analyzed the different scoring groups (IS1, 2 or 3).

Afterwards, we analyzed the probability of recurrence among papCPs, on the base of NpATM, and among adaCP, on the base of CpATM. No statistically significant correlation was observed (**table 2b**).

V Discussion

The CPs are benign epithelial tumors of the sellar region that arise from embryonic remnants of Rathke's pouch. They are rare neoplasms (incidence of 0.5 - 2.5 per 100,000 persons / year), most frequently in children representing, in this age group, 5-10% of intracranial tumors and the most frequent pituitary tumors (3). There are two subtypes, the adaCP and the papCP, which differ for histological and etiopathogenetic features, for the biological behavior and clinical outcome. The papCP is a fairly uncommon cancer, occurs almost exclusively in adults and has a favorable prognosis. The adaCP, the most common subtype, shows two peaks of onset, the first in children (5-15 years) and second in adults (45-60 years) and despite being a histologically benign tumors (grade I sec. WHO), shows a locally aggressive behavior that is manifested by a tendency to infiltrate the surrounding brain and vital neurovascular structures (3). There are no guidelines to treat these tumors and the choice is essentially based on two main therapeutic options, surgery and / or radiation therapy. The goal is to remove as much tumor as possible without impairment of the patients. However, despite the therapeutic efforts, the adaCP shows a tendency to recur frequently, whether as a result of the total removal or subtotal removal associated with radiotherapy (23% and 27%, respectively, in a post-operative period of five years) (22, 23). In recurrent cases the total removal is achievable in a percentage of cases considerably lower compared to primary tumors (0-25%) and is associated with increased mortality and morbidity (27). Therefore, even if patients with CP show a prolonged survival (> 85% after three years of

diagnosis), often have a poor quality of life because of the direct effects of tumor growth and the complications of the previous treatment.

For this reason it's important to identify tumors with more aggressive biological behavior. Currently we have no effective prognostic and predictive markers that can be used to discriminate cases of neoplasms characterized by greater biological aggressiveness and hence tendency to relapse. It is a topic debated, probably because the CPs are rare tumors whose pathogenesis is still unclear; in fact, the role of the Wnt pathway / beta-catenin in the pathobiology of adaCP (40-54) is well documented, whereas the emerging role of the stem cell is still controversial.

In this regard the most recent works (23, 49, 54, 58) suggest the presence of a minority of cells with stem cell phenotype in adaCP. From these cells originate clusters of neoplastic cells that are crucial for tumor growth and survival, by acting, probably, the production of a wide range of growth factors, chemokines, cytokines and their receptors (54). These clusters are variables for quantity, often show a "whorl-like" morphology, are in a state not proliferating (not express Ki-67) and, phenotypically, are characterized by the expression of the aberrant nuclear beta-catenin. The expression of stem cell markers in these clusters generated unclear and discordant data (49, 54). In the scientific literature beta-catenin and stem cell markers are widely studied in CP as etiopathogenetic factors but not for prognostic and predictive markers. The papers addressing the topic are few and often quite inaccurate. For example, Li et al (66), in a retrospective study of fifty CP, correlate the membrane expression of beta-catenin with a poor prognosis, defining the pattern of expression as "aberrant", contrary to the data, now known, reporting that the aberrant

expression of the protein is within the nucleus; Cao et al (67) studied the immunohistochemical expression of beta-catenin and p63 in a large enough group of sixty-six CP, comprising fifty-one adaCP and fifteen papCP, showing that the nuclear accumulation of beta-catenin is a morphological feature of the adamantinomatous subtype. Finally, in a more recent work, Esheba et al (68) evaluated the immunohistochemical expression of beta-catenin in a retrospective study of twenty CP, reporting that nuclear expression of aberrant beta-catenin is useful for the differential diagnosis between the two histological types of CP. Also in this work the clinical data, in particular those relating to the follow-up, are rather weak and shows no prognostic correlation. In 2013 Prieto et al (36) described a case of adaCP characterized by the rapid onset of tumor recurrence following surgery, performing a detailed review of the literature regarding the potential predictive factors of tumor recurrence. In this work epidemiological (age and gender of the patients) morphological, both macroscopic (tumor size, location, consistency, adhesion and invasion of surrounding structures) and microscopic (histological type, presence of clusters with "whirl-like" morphology, peri-tumoral reactive gliosis) and immunophenotypic (Ki-67 and p53) features were evaluated. The conclusion of this paper is that there is no consensus on the factors that may influence cancer recurrence, however, the only parameters that could suggest a closer follow-up are the presence of a marked reactive gliosis, a high value of Ki-67 and increased expression of p53.

Hölsken et al (23) evaluated the expression of stem cell markers (CD133 and CD44), both by immunohistochemistry and by real time PCR, in a group of thirty-three CP, which include eight papCP and twenty five adaCP; it was carried out, also, a correlation with the mutational status of

CTNNB1 and then with the aberrant nuclear expression of beta-catenin. The results obtained were the prevalent expression of CD44 in adaCP and CD133 in papCP and the co-expression, in the adaCP, of both stem cell markers in clusters of neoplastic cells characterized by the accumulation of nuclear beta-catenin. The interesting aspects of this work are the following: (I) for the first time was observed the expression of CD133 in human CP; it is well known that CD133 + cells are less sensitive to radiotherapy, therefore the expression of such markers may be significantly related to the onset of relapses in the CP; (II) it was demonstrated the expression of stem cell markers in the cells cluster characterized by the accumulation of nuclear beta-catenin, suggesting the presence of a "niche" of CSC involved in the pathogenesis of CP (69-70).

Based on the above, we decided to conduct a immunohistochemical study in a group of CPs, with the aim to identify potential prognostic and predictive markers selecting cases more aggressive and, therefore, at greater risk of recurrence.

We performed a retrospective study of 45 CP removed by endoscopic endonasal transphenoidal approach. The cases were divided into groups based on clinical outcome (presence or absence of tumor recurrence).

In 38 cases the diagnosis was of adaCP and in 7 cases it was of papCP. Of 4 recurrent cases only the primary tumor tissue was included for statistical evaluation; in 9 cases only the tissue of the recurrence was available (8 adaCP and 1 papCP). Therefore 13 cases were considered recurrent tumors and 32 not.

In this study it was then performed an immunohistochemical evaluation of the expression of β -catenin, the stem cell markers CD133 and CD166, pATM and rate of proliferation (Ki-67)

In our series, we observed that CD166 was expressed in CPs and it showed a membrane signal whose immunoscore correlated with β -catenin, Ki67 and recurrence risk. It remains unclear whether it behaves as a stem cell marker in CPs; though, it seems to spare the whirl-like cell clusters in adaCPs, where there should be the stem cell niches. However, regardless of its role in stemness, it can be of help, together with β -catenin, to predict recurrences in CPs. In our cohort CD133 was expressed in both histotypes, while it was described to be almost detectable in papCPs (11). The potential role of Ki67 as prognostic marker was confirmed. A cut-off of 5% was established as a valid tool to effectively distinguish highly proliferative forms from those with a low rate of proliferation. The Labeling Index was calculated on cells other than those of the basal layer where a high proliferative activity was expected.

As well, pATM expression was assessed in the attempt of identifying potential factors that can affect radiosensitivity in CPs. This kinase has a specific role in the DNA double-strand breaks repair, that is exercised in the nucleus, and has a cytoplasmic function that is related to autophagy (32).

In our series, we observed mainly a nuclear signal in papCPs and a cytoplasmic signal in adaCPs; this allowed us to consider that pATM different expression patterns in CP subtypes might be used as additional diagnostic and prognostic tool. No predictive role of recurrence was observed for pATM, neither cytoplasmic nor nuclear.

The prevalent cytoplasmic localization of pATM in adaCPs may be the effect of a different pathogenetic role of this kinase in the two histotypes. Further studies are needed to clear this aspect.

VI Conclusions

Finally, we suggest to use a panel of immunohistochemical markers including beta-catenin, Ki-67 and CD166, to identify *ab initio* cases of CPs with poor prognostic outcome, and therefore at greater risk of recurrence. To evaluate the expression of beta-catenin and CD166 may be useful to apply a score of immunoreactivity considering the intensity and the proportion of positive tumor cells; for the Ki-67 we suggest a cut-off value of 5% for distinguishing the low neoplasms from those with a high proliferative rate.

The identification of these markers is mandatory in order to draw prognostic evaluation of CPs (biological and clinical behavior) also in regards of recurrence risk of these lesions, therefore defining a more appropriate treatment strategy, with post-operative additional treatments.

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VIII Iconography

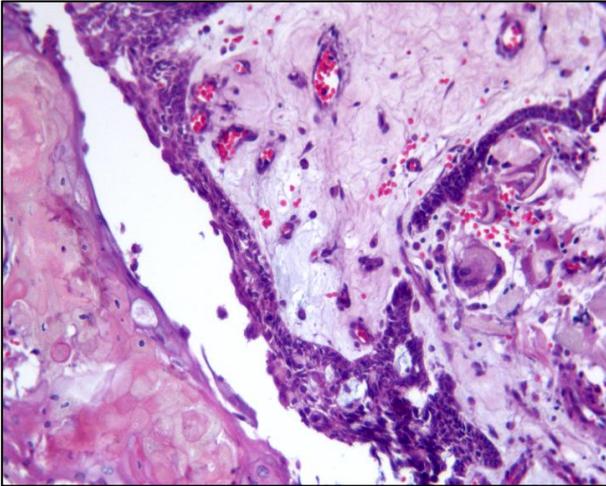


Figure 1. *AdaCP: cystic space lined with squamous epithelial cells mixed with nodules of "wet keratin" (E/E, 20x.)*

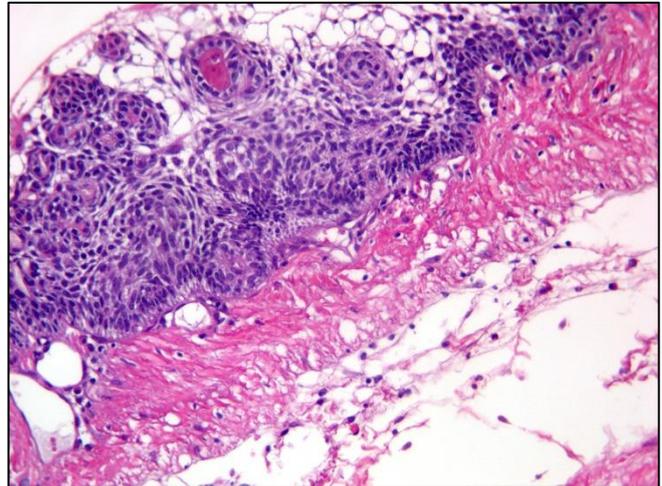


Figure 2. *AdaCP: clusters of neoplastic cells with "whirl-like" morphology (E/E, 20x).*

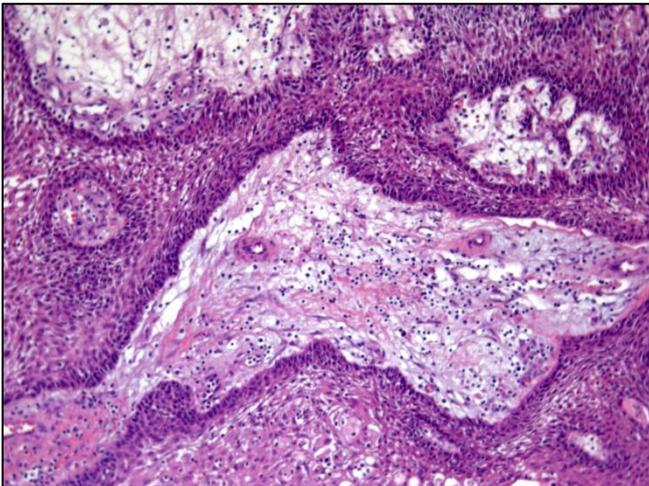


Figure 3. *PapCP: monomorphic proliferation of well-differentiated squamous epithelial cells arranged in lax pseudopapille (E/E, 10x).*

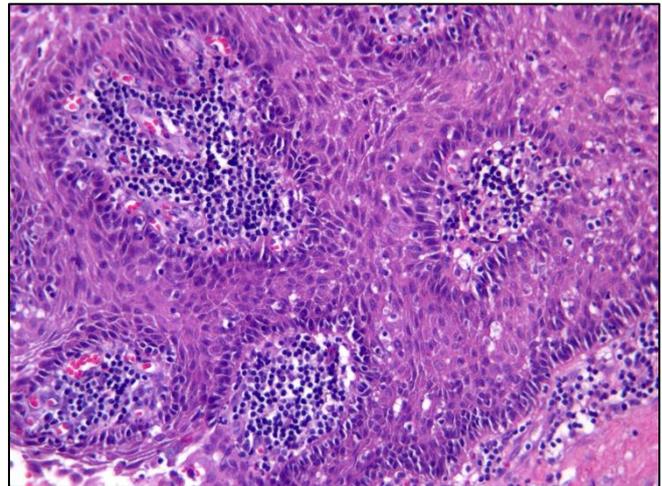


Figure 4. *PapCP: chronic inflammatory infiltrate in the axis of the papillae (E/E, 20x).*

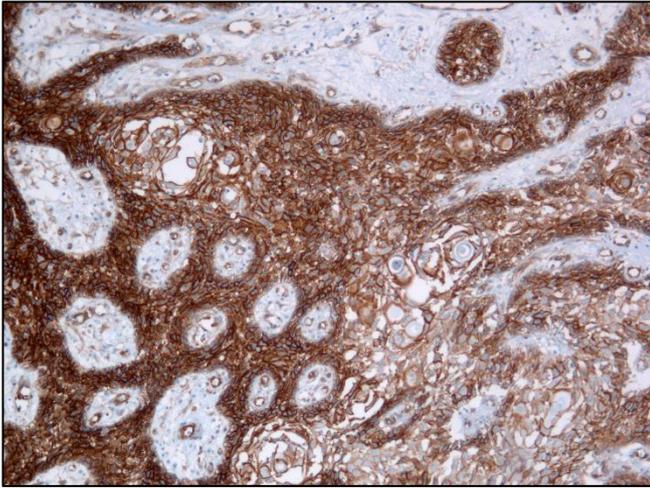


Figure 5. Immunohistochemical staining for beta catenin: immunoreactivity negative/ low in no recurrent adaCP

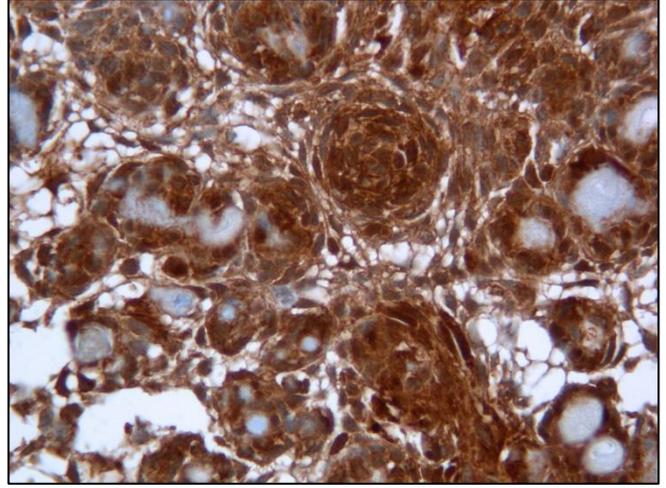


Figure 6. Immunohistochemical staining for beta-catenin: immunoreactivity moderate/high in recurrent adaCP

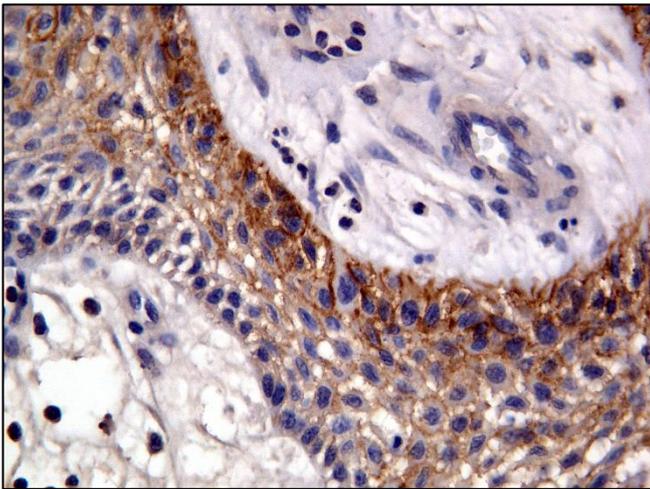


Figure 7. Immunohistochemical staining for CD166: immunoreactivity negative/low in not recurrent adaCP

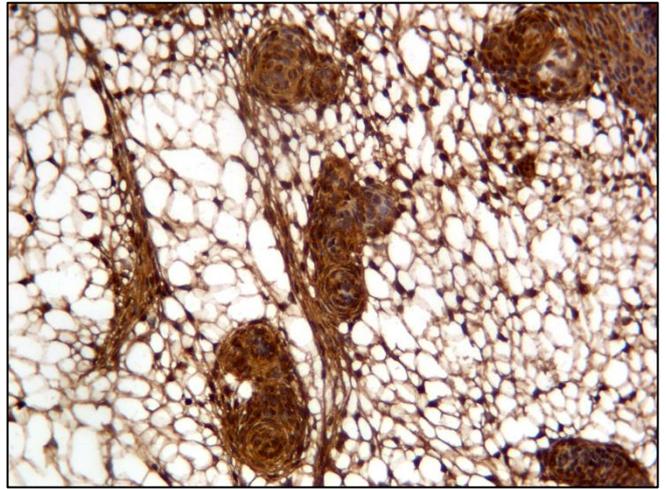


Figure 8. Immunohistochemical staining for CD166: immunoreactivity moderate/high in recurrent adaCP

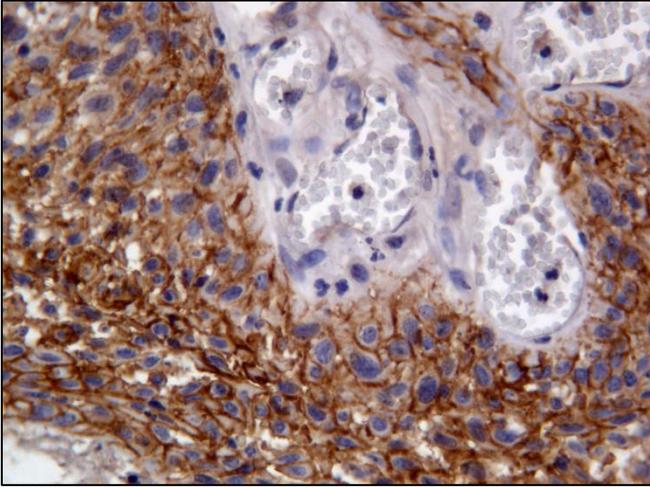


Figure 9. *Immunohistochemical staining for CD133: immunoreactivity moderate/high in recurrent papCP*

Table 1. Clinico-pathologic data

	Histotype [#]	Gender	Age	β-catenin*	CD133*	CD166*	Ki67	NpATM	CpATM	RT
1	ada prim	M	51	2 (1x2)	0	1(1x1)	2%	0	2	no
2	ada	M	61	0	0	0	3%	1	2	no
3	ada	F	13	1 (1x1)	1(1x1)	2(1x2)	2%	2	3	no
4	ada	F	2	2(1x2)	0	1(1x1)	10%	0	3	yes
5	ada	F	66	0	1(1x1)	0	2%	0	1	no
6	ada	M	9	0	0	0	1%	0	0	yes
7	ada rec	M	9	9(3x3)	4(2x2)	4(2x2)	25%	1	0	yes
8	ada rec	F	4	2(2x1)	3(3x1)	3(3x1)	--	0	2	yes
9	ada	F	15	1(1x1)	1(1x1)	1(1x1)	5%	1	1	no
10	pap rec	M	58	0	0	2(1x2)	6%	2	2	no
11	ada rec	M	63	6(2x3)	2(2x1)	6(3x2)	15%	0	2	yes
12	ada rec	M	51	2(1x2)	4(2x2)	4(2x2)	5%	0	1	yes
13	pap	F	47	0	6(3x2)	4(2x2)	6%	3	0	yes
14	pap	M	57	0	4(2x2)	4(2x2)	5%	3	0	no
15	ada prim	M	67	4 (2x2)	3(3x1)	3(3x1)	10%	0	0	yes
16	ada	M	48	0	0	0	2%	0	1	yes
17	ada rec	M	66	4(2x2)	2(2x1)	3(3x1)	7%	0	0	yes
18	ada	M	64	0	0	2(2x1)	3%	0	0	no
19	pap	M	27	0	4(2x2)	4(2x2)	--	2	0	no
20	ada	M	32	3(1x3)	1(1x1)	2(1x2)	2%	2	0	no
21	ada	M	57	2(2x1)	1(1x1)	0	3%	0	2	yes
22	ada	M	53	2(1x2)	0	1(1x1)	6%	2	0	yes
23	ada	F	43	2(2x1)	0	0	1%	3	2	yes
24	ada	F	36	0	--	0	5%	0	3	yes
25	ada prim	M	62	0	0	1(1x1)	15%	2	0	yes
26	ada	F	30	0	--	--	--	1	1	yes
27	ada prim	M	10	4(2x2)	0	4(2x2)	20%	2	2	no
28	ada	M	63	2(2x1)	--	--	20%	1	0	no
29	pap	M	49	0	0	2(1x2)	15%	2	0	no
30	ada rec	F	12	4(2x2)	3(1x3)	4(2x2)	n.v.	0	0	yes
31	ada	F	68	4(2x2)	0	6(3x2)	10%	1	3	no
32	ada	M	28	1 (1x1)	--	--	3%	2	0	yes
33	ada	M	77	0	--	--	2%	3	0	no
34	ada	F	52	0	--	--	4%	3	2	yes
35	ada rec	M	12	3(1x3)	0	4(2x2)	10%	0	1	no
36	ada	M	59	1 (1x1)	--	--	18%	2	0	no
37	ada	M	45	2(1x2)	--	--	8%	1	0	no
38	pap	M	42	2(1x2)	--	--	3%	2	0	yes
39	ada	M	42	4(2x2)	--	--	6%	0	1	no
40	ada	F	76	6(3x2)	--	--	15%	3	0	no
41	ada	M	39	0	--	--	20%	0	2	no
42	pap	F	50	0	--	--	7%	1	0	yes
43	ada	M	58	6(2x3)	--	--	15%	0	1	no
44	ada rec	M	21	4(2x2)	4(2x2)	3(1x3)	7%	1	0	yes
45	ada	F	54	2(1x2)	--	--	5%	3	1	no

*Immunoscore= intensity score x proportion score; # ada prim is the primitive tumor whose recurrence was not inserted in this table; ada rec and pap rec are tissues from the recurrent tumor, whose primitive was not available. RT: radiotherapy

Table 2a. Examination of correlation between clinical data and immunohistochemical scores

Variables	All cases N=45 (%)	B-catenin IS		p value	All cases N=30 (%)	CD133 IS		p value
		0-2	≥3			0-2	≥3	
Gender				0.4917				1.0000
Males	30 (67)	20 (62.5)	10 (77)		21 (70)	15 (71)	6 (67)	
Females	15 (33)	12 (37.5)	3 (23)		9 (30)	6 (29)	3 (33)	
Age				0.4113				1.0000
≤ 18 y	9 (20)	5 (16)	4 (31)		9 (30)	6 (29)	3 (33)	
> 18 y	36 (80)	27 (84)	9 (69)		21 (70)	15 (71)	6 (67)	
Histotype				0.0895				0.1432
adaCP	38 (84)	25 (78)	13 (100)		25 (83)	19 (90)	6 (67)	
papCP	7 (16)	7 (22)	0 (0)		5 (17)	2 (10)	3 (33)	
Recurrence				0.0039				0.1232
Yes	13 (29)	5 (16)	8 (62)		13 (43)	7 (33)	6 (67)	
No	32 (71)	27 (84)	5 (38)		17 (57)	14 (67)	3 (33)	
Variables	All cases N=31 (%)	CD166 IS		p value	All cases N=41 (%)	Ki67 L.I.		p value
Gender		0-2	≥3			0-2	≥3	
Gender				1.0000				0.4926
Males	21 (68)	11 (65)	10 (71)		29 (71)	12 (63)	17 (77)	
Females	10 (32)	6 (35)	4 (29)		12 (29)	7 (37)	5 (23)	
Age				0.6927				1.0000
≤ 18 y	9 (29)	4 (31)	5 (36)		7 (17)	3 (16)	4 (18)	
> 18 y	22 (71)	13 (69)	9 (64)		34 (83)	16 (84)	18 (82)	
Histotype				0.6358				0.6681
adaCP	26 (84)	15 (88)	11 (79)		35 (85)	17 (89)	18 (81)	
papCP	5 (16)	2 (12)	3 (21)		6 (15)	2 (11)	4 (19)	
Recurrence				0.0040				0.0385
Yes	13 (42)	3 (76)	10 (71)		11 (27)	2 (10)	9 (41)	
No	18 (58)	14 (24)	4 (29)		30 (73)	17 (90)	13(59)	

Table 2b. Examination of correlation between clinical data and immunohistochemical scores

Variables	All cases N=45 (%)	NpATM IS		p value	All cases N=45 (%)	CpATM IS		p value
		0	1-3			0	1-3	
Gender				0.7477				0.0574
Males	30 (67)	13 (72)	17 (63)		30 (67)	18 (81)	12 (52)	
Females	15 (33)	5 (28)	10 (37)		15 (33)	4 (19)	11 (48)	
Age				0.4487				0.4591
≤ 18 y	9 (20)	5 (28)	4 (15)		9 (20)	3 (14)	6 (26)	
> 18 y	36 (80)	13 (72)	23 (85)		36 (80)	19 (86)	17 (74)	
Histotype				0.0313				0.0470
adaCP	28 (80)	18 (100)	20 (74)		38 (84)	16 (73)	22 (96)	
papCP	7 (20)	0 (0)	7 (26)		7 (16)	6 (27)	1 (4)	
Recurrence*				1.0000				1.0000
Yes	1 (14)	0 (0)	1 (17)		12 (32)	8 (32)	4 (31)	
No	6 (86)	1 (100)	5 (83)		26 (68)	17 (68)	9 (69)	

* recurrence was evaluated only among papCP for NpATM and only among adaCP for CpATM

