Original Paper

Clinical, histological, immunohistochemical and statistical aspects in malignant nasopharyngeal tumors

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ABSTRACT: Various types of cancer can be found at a nasopharyngeal level, and most of them are carcinomas (NPC=nasopharyngeal carcinoma). The most frequent and clinically important carcinoma is the undifferentiated one, known as UCNT. It is localised form, it is a very radiosensitive and radiocurable type of cancer, but strong emphasis has also been placed on its high chemosensitivity. Actually, its connection to the Epstein Barr virus (EBV) drew numerous researchers' attention. The use of immunohistochemical markers in the study of malignant nasopharyngeal tumours for diagnosis purposes and in order to determine the exact nature of undifferentiated tumours for scientific purposes and for the assessment of the prognosis thus becomes absolutely imperative.

KEYWORDS: *Malignant nasopharyngeal tumors, EBV antibodies, immunohistochemistry, histology, geographical display.*

Introduction

Benignant and malignant nasopharyngeal tumors, however rare they may be, are characterised by insidious, oftentimes atypical early stages, which makes them difficult to diagnose. Their diagnosis is therefore generally tardive, because the cavum is difficult to explore from a clinical point of view and the lesions are not visible to the bare eyes, and because the early stages are often paucisymptomatic, which makes an accurate diagnosis difficult to give in a timely fashion. Symptomatology can often have one of the five different forms: nasopharyngeal signs, auricular signs, neuralgic signs, ganglionary signs or ocular signs (which are less frequent).

A delay in establishing the correct diagnosis can account for the fact that therapeutic attitudes are often palliative. On the other hand, the occurrence of malignant tumors at this level has increased lately.

By corroborating clinical, histopathological and immunohistopathological data we can contribute to an earlier diagnosis of nasopharyngeal tumors, enabling us to start treatment faster. This can lead to either healing the patients, making their lives longer or avoiding permanent functional and aesthetical damage.

Materials and methods

Routine colorations are important, but immunohistochemical determinations are also necessary and useful in order to give a precise diagnosis, to determine the origin of poorly differentiated malignant cells, to perform tumor grading, and to collect prognosis information. The tumor fragments that were used for the histological diagnosis were fixed in a 10% formalin solution, cut after paraffin treatment in 4-micron thick cups and stained with haematoxylin and eosin.

The immunohistochemical study was performed on 26 cases (two non Hodgkin lymphomas, a follicular lymphoma, an esthesioneuroblastoma, a fibrosarcoma, a malignant tumor of the peripheral nerve and twenty carcinomas) of malignant nasopharyngeal tumors which were initially studied from a histopathological point of view. The objectives were: to confirm the malignant tumor diagnosis, to determine the histogenesis (the type of malignant neoplasm) and to provide a differential diagnosis as compared to other malignant tumors.

The immunohistochemistry technique in itself consisted of a standard algorithm, with certain variations according to the antibodies that were used (TABLE 1)

Table 1: The antibodies used in this study

Antibody	Epitope / marker	Dilution	Antigenic reaction	Source
CD20	L26	1:100	3 cycles citrate buffer	DAKO
CD79a	SP18	1:300	7c-citrate	THERMO
CD56	1B6	1:100	5c-citrate	LEICA
PCNA	PC10	1:400	7 cycles citrate buffer	DAKO
Ki-67	MIB-1	1:20	7 cycles citrate buffer	DAKO
AE1/AE3	NCL-L-AE1/AE3	1:100	5c citrate	LEICA
P53	DO-7	1:25	5c citrate	DAKO
EMA	E29	1:50	5c citrate	DAKO
EGFR	E-30	1:200 CSA II		DAKO
Actin	1A4	1:50	3c citrate	DAKO
S100	Polyclonal	1:500		DAKO
Vimentin	V9	1:100	5c citrate	THERMO
NSE	BBS/NC/VI-HI4	1:200	7c citrate	DAKO
EBV LMP-1	CS 1-4	1:100	sodium citrate pH 6	DAKO
CK 34betaE12	34betaE12	1:50	sodium citrate pH 6	DAKO

Results

The study consisted of 107 patients with malignant tumors (59,28%), most of whom suffered from squamous cell carcinoma. Depending on the origin, 57 cases (53%) originated from the rural area and 50 cases (47%), originated from the urban area. Depending on the sex, malignant tumors occurred 2,34 times more frequently in men than in women (75/32). As for the age, the peak was during the 50-59 years old decade.

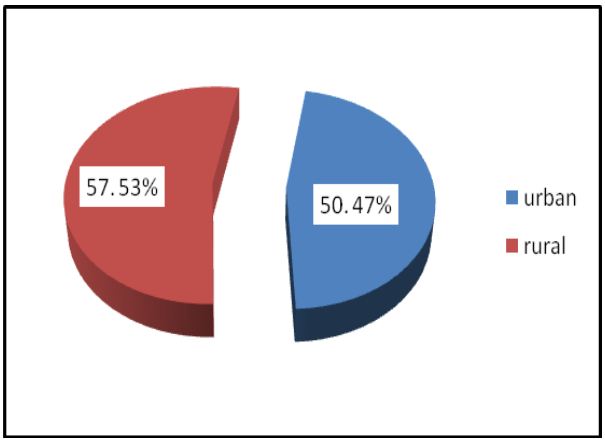

Fig.1. The distribution of the group depending on the origin

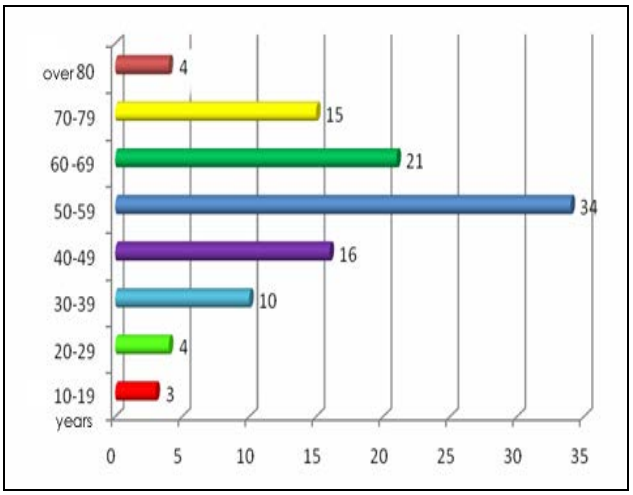

Fig.2. The distribution of the group depending on the gender

91,58% of the malignant tumors were carcinomas, 6,6% of them were lymphomas (4 cases of diffuse large B-cell malignant non Hodgkin lymphomas and two cases of follicular lymphoma, with an even proportion of men and women), 1,1% malignant peripheral nerve sheath tumor (MPNTS), 1,1% fibrosarcomas and 1,1% esthesioneuroblastoma .

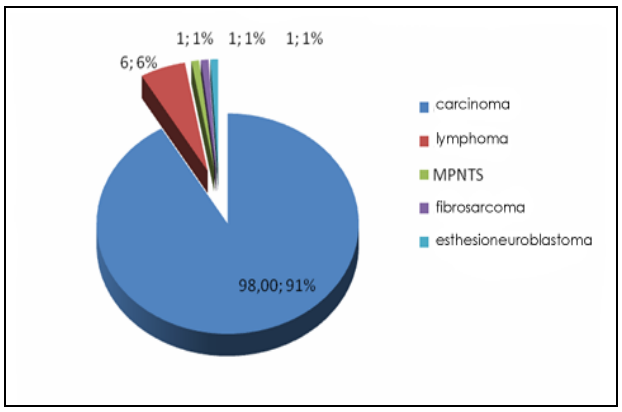

Fig.3. The distribution of the casuistry depnding on the tumor type

Carcinomas can be of three histological types. The first type, the keratinized one, occurred in 27 cases (27,3%), with several subtypes (an in situ carcinoma, two microcarcinomas, eight well differentiated squamous carcinomas, ten moderately differentiated squamous carcinomas and six poorly differentiated squamous carcinomas). The second type, the nonkeratinized one, occurred in 15 cases (15,6%), while the third type, the undifferentiated one, occurred in 56 cases (57,1%).

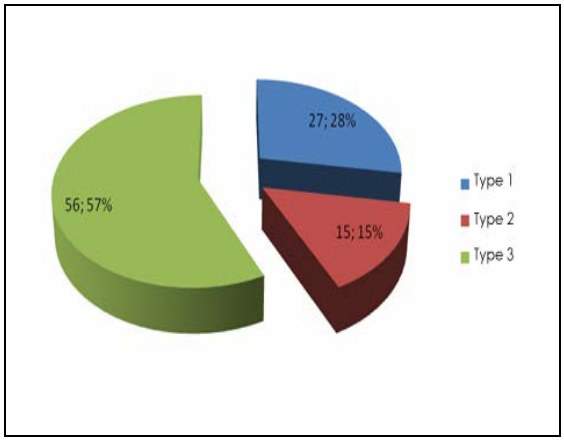

Fig.4. The distribution of the carcinomas

Although the molecular basis of the pathogenesis of the nasopharyngeal carcinoma are not yet clear, it has been suggested that several factors appear: the EBV infection, environmental and genetic factors which are of major importance for the carcinogenesis [1]. During the development and progress of this condition, a series of genetic abnormalities are accumulated, which, alongside with the synergic effect of the EBV infection and the environmental factors can act to modify the normal cell functions and the signalling pathways [2].

The Ebstein Barr virus (EBV) was studied by means of an immunohistochemical examination and in situ hybridization in ten cases and its presence was revealed in nine of them. The etiological association between the undifferentiated carcinoma and the Epstein-Barr virus is known to have been confirmed in 75-100% of the cases.

The 1st type is the keratinized squamous cell carcinoma. At a microscopic level, it is characterized by the presence of unevenly distributed epithelial tumor cells with numerous intercellular bridges and a high level of keratin. The keratinized squamous cell carcinoma has several degrees of differentiation (ciscarcinoma, microcarcinoma, G1 well differentiated squamous cell carcinoma, G2 moderately differentiated carcinoma, G3 poorly differentiated carcinoma G3).

In the case of in situ carcinoma it has been noticed on the Hematoxylin and Eosin stained lamellae the presence of malignant epithelial protuberations represented by cells with nucleo-cytoplasmic tahicromasies or malignant epithelial proliferations with evidence of keratin pearls without going beyond the basement membrane (Figure 5a, b).

The well differentiated squamous cell carcinoma is characterised by the presence of malignant tumor cells in strings (polygon-shaped, similar to the cells of the epidermal layer, but presenting atypical characteristics), with an infiltrative character (the basement membrane is destroyed). The differentiation degree of the tumor is set by the cellular atypia and the production of keratin by the tumor cells. In keratinized squamous cell carcinomas, keratin gathers around the tumor strings as intensely eosinophilic concentric lamellae, called keratin pearls. The conjunctive stroma is reduced and infiltrated with lymphocytes. The inflammatory infiltrate in the stroma generally has a peritumor disposition, it is lymphoplasmacytic and it can vary in quantity, ranging from reduced, to moderate or to abundant. In the case of the ulcerated tumors, the inflammatory infiltrate is intratumoral and has a high level of polimorfonucleares (Figure 5c).

The moderately differentiated squamous cell carcinoma (G2) was diagnosed in ten cases and it was characterized by a small number of keratin pearls, incomplete keratinisation and persistence of the nuclei. The cellular mitosis and atypia are more frequent than in the well differentiated type (Figure 5d).

The poorly differentiated squamous cell carcinoma was characterized at a microscopically level by the fact that the tumors consisted of compact formations of cells of uneven shapes and sizes. There was no keratinisation or the existing one had a unicellular character, with a marked cellular atypia and numerous atypical mitoses. Sometimes it was also noticed the presence of microcarcinomal areas, at other times the tumor stroma was poorly represented or an abundant fibrocollagenous tissue with tumor pseudonodules (Figure 5e).

The 2nd type is the nonkeratinized carcinoma. At a microscopically level, the tumor cells have a high degree of pleomorphism, the have developing patterns such as epithelioid, fusiform, transitional, lymphoepithelial, with clear cells, anaplastic. There is frequently a combination of such patterns (Figure 5 f).

The 3rd type is the undifferentiated nasopharyngeal carcinoma, and it was identified in 56 cases. At a microscopically level the undifferentiated type presented solid strings with a syncytial aspect with large oval or round vesicular nuclei, prominent central nucleoli, poorly defined limits, a low quantity of cytoplasm, inflammatory infiltrate in 12 cases, trabecular disposition with intra and peritumor inflammatory infiltrate in 22 cases (Figure 5g). Immunohistochemical profile: in all cases the tumor cells showed a cocktail of cytokeratins (AE1/AE3) and EMA, and in 12 cases vimentin was positively identified. The prognostic information we have collected as a result of immunohistochemistry comes from indexes of cellular proliferation of the PCNA, Ki 67 and P 53 factors, as well as the EGFR as angiogenesis marker [3]. From an immunohistochemical point of view these carcinomas express the above mentioned cytokeratins as well as the 34beta E12, which we were able to notice in the ganglionary metastasis (Figure 1h).

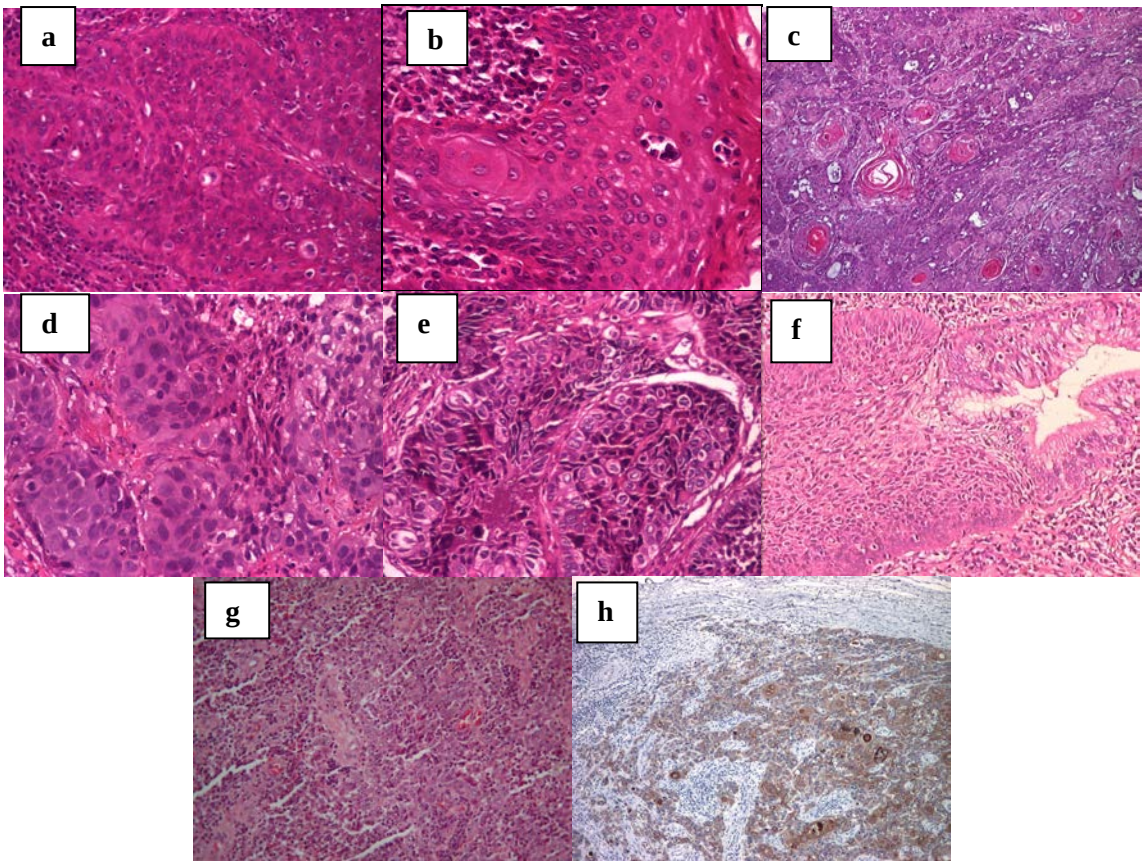

Fig.5. Different types nasopharyngeal carcinoma HE col.

The follicular lymphoma was characterized at a microscopic level by a nodular proliferation of small lymphocytes with poor cytoplasm, with incised nuclei with no nucleoli; among them it was noticed the presence of rare cells with eosinophilic cytoplasm, with vesicular nuclei and 1-2 nucleoli attached to the nuclear membrane. We were able to notice the absence

of germinative centres, with macrophages and tingible bodies and the absence of the mantle zone.

The esthesioneuroblastoma is an embryonal tumor with small, round, blue cells. At a microscopically level the tumor cells are displayed in strings of small, round, blue cells with a vague lobular pattern or nests displayed in a neurofibrillary matrix with the occasional formation of rosettes (Homer-Wright) as shown in Figure 6a.

In the cases we have studied a pleomorphic fibrosarcoma was diagnosed, characterized at a microscopic level by the proliferation of fibroblasts displayed in short fascicule with large cells with pleomorphic nuclei, some of which were multinucleated, with a dense collagen stroma (Figure 6b).

The peripheral nerve malignant tumor consists of dense cellular fascicule which alternate with myxoid areas. The strings of fusiform cells alternate with strings of round and epithelioid cells, with necrosis areas and cellular pleomorphism (Figure 6c). (FIGURE 6)

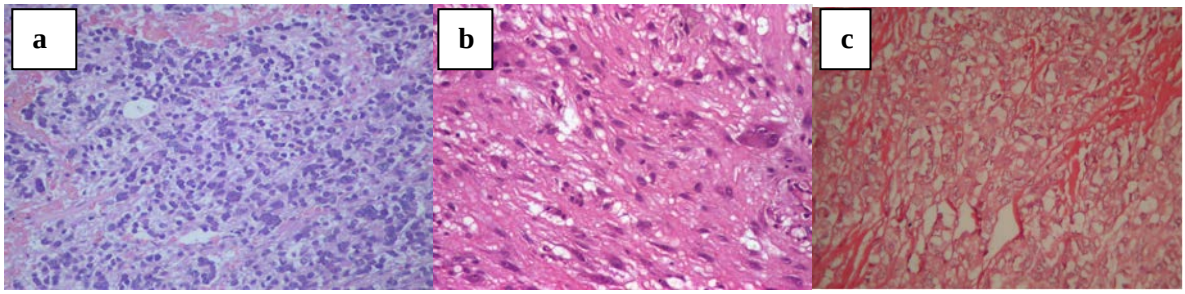

Fig.6. MPNTS HE col.

The large B cell diffuse lymphoma was characterized at a microscopic level by a monomorphous proliferation of large B lymphocytes with a clear eosinophilic cytoplasm and some cells with basophilic cytoplasm (Figure 7a).

From an immunohistochemical point of view, in the case of lymphomas, the tumor lymphocytes expressed CD20, CD79a, Ki 67 15% and showed no evidence of CD21, CD23, and D1cyclins (Figure 7b,c).

From an immunohistochemical point of view, in the case of the large B cell diffuse lymphoma, the tumor cells expressed more than 30% of CD20, Ki 67; they showed no evidence of CD5, CD23 (Figure 7d). (FIGURE 7)

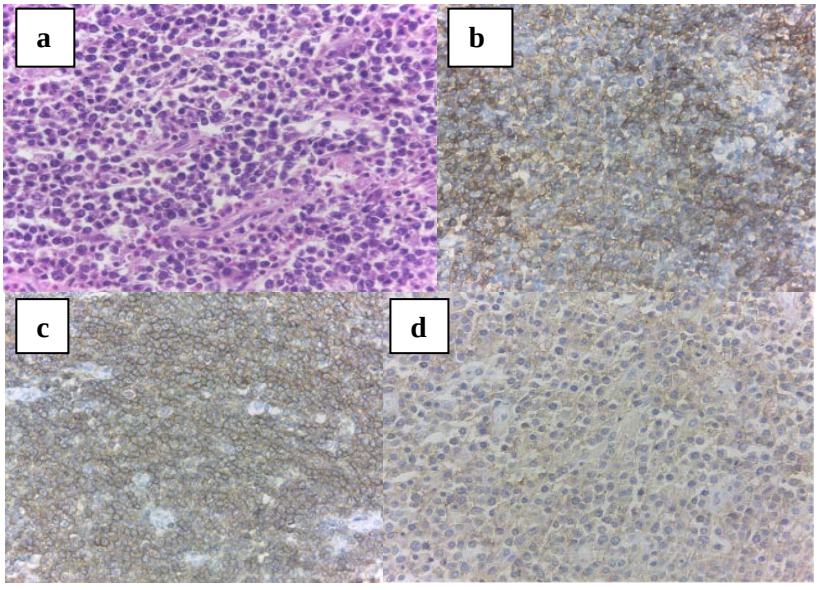

Fig. 7. Lymphomas IHC and HE col

From an immunohistochemical point of view, the tumor cells in the esthesioneuroblastoma expressed NSE, S-100, vimentin, Ki 67 (over 30%), as shown in Figure 8a, and the malignant tumor cells in the tumors of the peripheral nerve sheath expressed over 30% vimentin, S100 and Ki 67 and showed no evidence of actin (Figure 8b).

From an immunohistochemical point of view the tumor cells in the fibrosarcoma tested positive for vimentin and negative for alfa actin, S100 (Figure 8c) and AE1/AE3 (Figure 8d) (FIGURE 8)

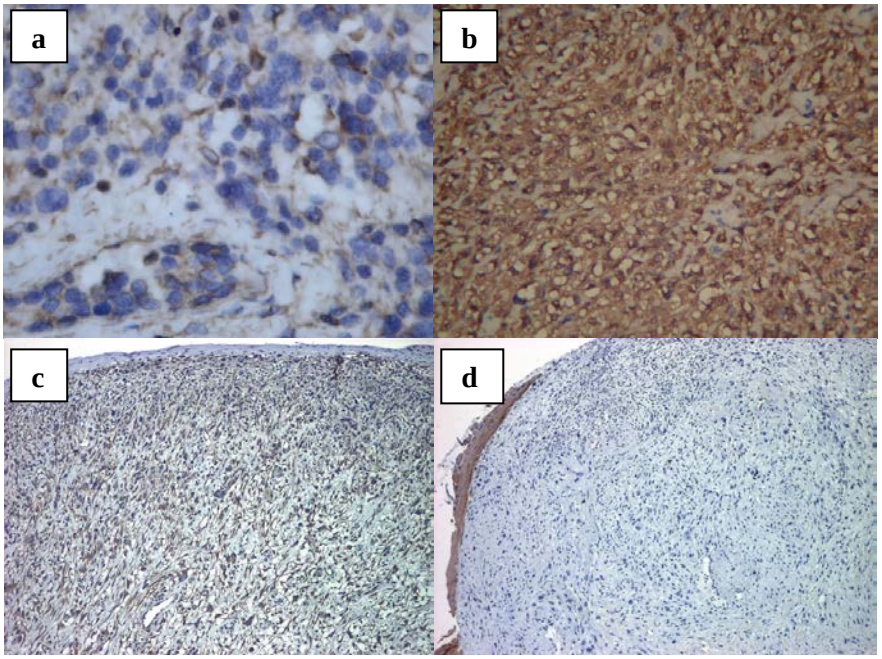

Fig.8. MPNTS IHC col.

Discussions

Considering their incidence level, the nasopharyngeal malignant tumors have an uneven global distribution, with areas such as south-eastern Asia or Eskimo Alaska where nasopharyngeal tumors can occur in 20-30 cases (up to 50 cases in the Gungdong province in southern China) in one hundred thousand inhabitants.

It could also be noticed the fact that in the case of the Chinese people who emigrated to areas with a low incidence level, the number of cases in the next generation decreased, but it was still 6 or 7 times higher than in the case of the population residing in low-risk areas, which can prove a certain genetic predisposition alongside with the Epstein Barr virus infection and the influence of the environmental factors (eating habits).

There is also a medium incidence area, in North Africa and in the Mediterranean countries, where 5-9 cases can occur in one hundred thousand inhabitants.

Low incidence areas are north America and western Europe, with a 0,5% incidence in one hundred thousand inhabitants.

In our study, most cases (91.85%) consisted of the nasopharyngeal carcinoma, a malignant tumor generated in the covering epithelium of the nasopharynx and which can be distinguished among other brain and throat tumors by its distinctive histopathological aspect, geographical and racial distribution, clinical characteristics and treatment.

The nasopharyngeal carcinoma is the main cause of death for south eastern Asian populations and for less of the north African populations [4] and it is approximately 18% of the malignant tumors of Asian populations [5]. It is a rare tumor in other parts of the world, where the incidence rates are generally lower than 1% in one hundred thousand inhabitants a year. In the USA, the nasopharyngeal carcinoma occurs in 7 cases in 100000 inhabitants [6], and in Egypt, the National Cancer Institute identified a 0,39% incidence rate in the entire malignant pathology [7].

The nasopharyngeal carcinoma has a bimodal distribution according to age: one peak in late

childhood and another peak between 50-60 years of age, with a M:W rate of 2:1. In our study we identified a 50-60 years of age peak, since the profile of the ENT section was an adult one, and we were unable to address young aged patients (only 3 cases were diagnosed for patients between 10 and 19 years old).

With this particular global distribution of the nasopharyngeal carcinoma, it is important to notice the existence of a universal histopathological classification for comparable and standardized diagnosis and therapies. However, since the beginning of the 20th century a series of classification schemes has been suggested, which has led to confusion. In 1978, the first international classification suggested by the World Health Organisation (WHO) classified the nasopharyngeal carcinoma in 3 subtypes, on account of the differentiation of the tumor cells [8]. The first subtype is characterized by keratinized squamous cell carcinomas with distinctive intercellular bridges and keratin production. The second subtype is the unkeratinised squamous cell carcinoma, with a varied cellular morphology: fusiform or polygon shaped. The third subtype is the undifferentiated squamous cell carcinoma with distinctive cytological characteristics, with poorly defined cells, reduced cytoplasm and visible nucleoli.

In low incidence areas such as north America, 25% of the patients who were diagnosed with nasopharyngeal carcinoma are of the first type, 12% are of the second type and 63% were of the third type, unlike the endemic areas where the distribution is of 2%, 3% and 95% [9]. In our study, the first type included 27,3%, the second type included 15,6% , and the third type included 57,1% (Chart 4).

The role of the EB virus in determining the occurrence of the nasopharyngeal cancer was discovered during a series of serological studies aimed at the Burkitt lymphoma, which showed a high level of antiEBV antibodies in patients who were diagnosed with rhynopharyngeal neoplasm. It was thus proved to be a connection between the EBV and the undifferentiated rhynopharyngeal carcinoma, with no influence of the geographical incidence area, unlike the Burkitt lymphoma, which tested negative for 80% of the cases which do not occur in Africa [10].

The EB virus is identified in the epithelial cells by means of two specific viral markers, also emphasized at the level of the B lymphocytes and of the cavum epithelial cells. The markers are represented by the viral genome as free DNA and by the nuclear antigen of the EB virus (EBNA).

The high level of the IgG and IgA antibodies used against the EB virus is characteristic to the patients suffering from undifferentiated rhynopharyngeal carcinoma, but their levels are low in those suffering from other types of air or digestive types of cancer or in healthy individuals. There are three types of IgG and IgA antibodies used against viral capsid (VCA), of the nuclear antigen (EBNA) and of the early antigen (EA) of the Ebstein Barr virus. The most important ones seem to be the IgG/EA and the IgA/VCA, the level of which is proportional with the specific stage in the evolution of the condition (their level decreases once the treatment has started). The high level of these antibodies after the treatment has started indicates a lack of efficiency and a continuation of the disease. Many researchers maintain that it has a high prognostic value.

By means of a series of tests performed on a large number of individuals in southern China, Ainsi Zeng proved that a high level of IgA/VCA antibodies indicates a risk of developing rhynopharyngeal cancer in the next 18 months which is between 80 and 100% higher than in the rest of the cases.

Even from the first visit to the doctor's, 62 of the patients with squamous carcinoma had one or both sided adenopathy, that is 63,26%, specific research mentioning between 60% and 70% of first visit adenopathy.

In our study, the 98 cases in the 2.3 million inhabitants in Oltenia, lead to a 0,42 incidence for one hundred thousand inhabitants, which is similar to the data in other specific studies.

From a clinical point of view, the anatomical situation of the rhynopharynx makes it difficult to explore, which can lead to a high possibility of not detecting it during a superficial clinical examination. Any neighbouring symptom – transmission hypoacusis and serous otitis, nasal obstruction and epistaxis, trigeminal neuralgia, pharyngeal paralysis, diplopia – require a detailed consult in the field of ENT and other specialties (neurology, ophthalmology). Any laterocervical adenopathy in the upper third on the anterior edge of the sternocleidomastoid muscle should be an indication of a rhynopharyngeal tumor process.

All patients were subjected to lung radiography (for the detection of lung metastasis), to cervical echography (in some cases abdominal echography also) for the

detection of any ganglionary metastasis, and to laboratory analysis for finding the level of the antiEBV antibodies. Computed tomography has been very helpful in establishing the local extension (the invasion of those structures neighbouring the rhynopharynx) or the distance metastasis, and nuclear magnetic resonance has enabled us to distinguish between the various types of inflammatory infiltrate and tumor processes and the invasion of the nervous structures in a more accurate way than by means of the CT [11].

All these investigations, alongside with the histopathological results and the correct TNM staging represent a necessary step before the initiation of the oncological treatment.

As a means of oncological treatment, chemotherapy has a radiosensitivizing role in reducing the rate of distance metastasis, but it can also be used in the case of recidivism or metastasis, in order to prolong the patients' lifespan and improve the quality of their lives. Irradiation is the basic treatment to be chosen to fight the tumor. Conventional roentgen therapy and transcutaneous telecobalt therapy are used on two lateral pretragal areas and two trans-maxilo-nasal areas in dosis of up to 8000 R. Regardless of the presence or absence of cervical adenopathy, the irradiation of ganglionary areas must be systematic and bilateral. A 5000 R dose and an extra 2500 R are administered on the invaded side. Cobalt therapy can also be used with 75 Gy dosis at the level of the tumor, but this procedure is difficult to use because of the vicinity of sensitive structures (eyes, nerves, and the ear). The side effects are disagreeable and sometimes almost unbearable. The most effective cytostatic drugs seem to be Adriamycin, Cysplatin and Bleomycin [12].

After the oncological treatment we noticed the disappearance of the rhynopharyngeal tumors, even in those cases which originally showed a complete invasion of the cavum, and the ganglionary metastases got to smaller dimensions. Lymphadenectomy was seldom necessary, and it was always performed after the oncological treatment on the remaining ganglionary lesions. The distance metastases were proportional to the size of the ganglionary ones, and therapeutic failure mostly occurred in the case of differentiated (radioresistant) tumors and in the case of local bone invasion. There has also been no sign of locations other than the original tumor, unlike the upper airways types of cancer, with a second localisation in 20% of the cases.

As for the survival rate, statistics mention 40% three-year survival and 20-30% five-year survival. The survival rate also depends on the histological nature of the tumor, on the presence or absence of skull base invasion and on the amplitude of the ganglionary metastases.

Conclusions

Our study dealt with 107 patients diagnosed with rhynopharyngeal neoplasm, the most frequent of which having been the squamous cell carcinoma, and the most frequent of the three histological types was the undifferentiated one (the 3rd type). The well differentiated squamous cell carcinoma was characterized by the presence of the strings of malignant tumor cells (polygonal shaped, similar to the cells of the epidermal layer, but with atypia), of an infiltrative nature (the basal membrane is destroyed), the differentiation degree of the tumor being set by the cellular atypia and the production of keratin by the tumor cells. The cytokeratins (AE1/AE3), EMA and vimentin were tested positive, and the PCNA, P 53, Ki 67 and EGFR as an angiogenetic marker had a prognostic value, indicating a tumor progress [13]. From an immunohistochemical point of view, in the ganglionary metastases these carcinomas express the above mentioned cytokeratins as well as the 34beta E12. It can be noticed that in all the types of tumors we have studied the Ki 67antigen, which is a marker of the cellular cycle and of the cellular proliferation is a negative prognostic factor, because the higher the number of tumor cells with a positive Ki 67 is, the more important tumor progress is. With the exception of lymphomas, vimentin was another negative marker for the tumors we have studied.

Patients with nasopharyngeal carcinoma show a high level of IgG/EA and IgA/VCA antibodies as compared to the EB virus, which constitutes a prognostic factor for the diagnosis and evolution of the condition in the eyes of many authors.

As compared to the data in other specific studies related to the incidence of this condition, the results in our study are insignificantly different, which enables us to maintain that the results are superposable.

Our immunohistochemical study emphasizes the importance of a detailed immunohistochemical examination for a correct diagnosis, and for its prognostic value[14].

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