Immunologic and cytogenetic markers expressed in non-Hodgkin lymphoma of head and neck

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Abstract
Very often, the first doctor who examines a patient with malignant lymphoma is an ENT specialist, because non-Hodgkin lymphoma is five times more frequent than Hodgkin disease in the head and neck region. Approximately 25% of extranodal lymphoma occurs in the head and neck and extranodal presentation is twice as frequent as nodal presentation. This paper presents a study of the patients from ENT, Head & Neck Surgery Clinic of Coltea Clinical Hospital, Bucharest, Romania, diagnosed with malignant lymphoma. We developed a specific scheme for collecting data about patients, together with pathology details, immunology and cytogenetic markers. We tried to establish a relation between immunologic and cytogenetic markers and the clinical evolution of non-Hodgkin lymphoma. For this study, we analyzed data regarding CD10, CD5, CD20, Bcl-2, Bcl-6, Ki67 expression obtained from 58 patients with follicular lymphoma. An attempt was made to correlate the presence of certain immunohistochemical and cytogenetic markers with the evolution and aggressiveness of the disease, and we can say that Bcl-2 is positive in all tumor subtypes, being associated relatively frequently with CD5 expression, and is a marker of poor prognosis, while Bcl-6 is positive especially in the tumor forms associated with the predominance of small cells and is a marker of favorable prognosis.

Keywords: malignant lymphoma, cytogenetic markers, extranodal involvement.

Introduction
Out of the numerous etiologies, which can express as swelling of the cervical lymph nodes, we have chosen an issue, which is commonly encountered within the ENT Department of the Coltea Clinical Hospital, Bucharest, and we have based this choice on the collaboration with the Hematology Department. The issue in question is the ENT expression of malignant lymphoproliferations.

Malignant lymphomas represent the second most frequent cause of head and neck adenopathies. They usually occur in the head and neck areas with adenomegalgy but also with extranodal sites (Waldeyer ring, rhinopharynx, thyroid, paranasal sinuses). However, while Hodgkin disease occurs especially in the lymph node chains, non-Hodgkin lymphoma is more frequently encountered in the extranodal sites.

The diagnosis of certainty is made based on the histological examination of either the nodes obtained by means of neck dissection or the biopsies from the extranodal sites, completed by immunohistochemical and cytogenetic tests.

During the past years, the immunophenotypic and genotypic analysis with the aid of monoclonal antibodies – that carry specificity for detecting cellular antigens (surface, cytoplasmic or nuclear) in cellular suspensions or frozen sections – as well as the new molecular biology techniques have allowed the identification of the malignant population that proliferates monoclonally and the recognition of the morpho-functional stages of differentiation at the level of a cellular line [1–3].

Malignant non-Hodgkin lymphomas involve Waldeyer’s ring far more frequently, leading to deglutition impairment (tonsillar lymphoma or mediastinal periesophageal adenopathy) [4]. Also, they more frequently involve the rhinopharynx, which in turn leads to a debut of nasal obstruction, loss of hearing or nosebleed. Splenomegaly is found in approximately 40% of patients with non-Hodgkin lymphoma, sometimes associated with hypersplenism and pancytopenia. The impairment of the spleen in Hodgkin disease closely precedes the hepatic involvement. Central nervous system (CNS) determinations especially occur in lymphomas bearing a high degree of malignancy, particularly the lymphoblastic ones. In malignant lymphomas other determinations may occur, such as renal, pulmonary, pleural, osseous, tegumentary, of the orbit, testicles, breast a.s.o. very significant cytopenia is rare, and it occurs solely in the case of bone marrow involvement. 40–70% of the patients with low-
malignancy lymphomas may present with peripheral lymphocytic discharge whereas only 10% of cases with aggressive lymphoma present with a leukemic phase, in which situation the prognosis is poor. General symptoms are as follows: persistent fever, profuse sweating, weight loss, asthenia. When these are present, it usually means the disease is in an advanced stage.

There are no effective screening methods, nor are there effective ways of identifying the population at high risk for developing lymphomas.

In spite of the progress made in the field of immunologic, cytogenetic and molecular techniques, the diagnosis and histological classification of lymphomas remains problematic for clinicians and pathologists.

In follicular lymphomas, the \((14;18)\) translocation has been discovered – it is considered specific and diagnostic for this histological type. It is encountered in 85% of follicular lymphoma cases and in 28% of aggressive lymphoma cases. In this translocation, the Bcl-2 gene approaches the gene for the Ig heavy chain. The Bcl-2 gene codifies a protein capable of inhibiting apoptosis, thus the neoplastic cells gain a longer duration of life, which in turn leads to their local accumulation.

\begin{itemize}
\item \text{Materials and Methods}
\end{itemize}

\textbf{Work hypothesis:} lymphomas represent a model for the study of the role of oncogenes in cellular physiology.

On a global scale, the role of clonal molecular markers in the tumor growth and development represents one of the most modern investigation fields. At present, there are still unclear aspects regarding the involvement of these factors in the disease prognosis and the therapeutic conduct, as follows:

\begin{itemize}
\item The cytogenetic \((14;18)(q32;q21)\) anomaly – detectable by PCR in 80% of follicular lymphoma cases and characterized by the increased expression of the Bcl-2 gene with subsequent inhibition of apoptosis – is necessary but not sufficient \([3, 5]\);
\item The role of the Bcl-6 expression in the clinical evolution of patients with lymphoma is still unclear \([3, 6, 7]\);
\item The proliferation index measured by the immunohistochemical determination of Ki67 is correlated with the tumor’s degree, but it is not clear in which way it is correlated with the clinical aggressiveness.
\end{itemize}

In the study that we conducted, we used retrospective data of patients diagnosed with malignant proliferations by means of histopathologic examination of the lymph node obtained by neck dissection within the ENT Department of the Coltea Clinical Hospital, Bucharest, between 2004–2010.

A chart for the patient’s identification has been elaborated, as follows:

\begin{enumerate}
\item Identification data: patient’s initials, sex, age, date of diagnosis, profession, number of siblings.
\item Significant history: oncologic, toxic, cardiac, infectious, renal, hepatic, neurologic, other.
\item Clinical and laboratory assessment:
\begin{itemize}
\item Clinical: ECOG performance status, weight loss, serum proteins, albumin, hemoglobin, mean red blood cell volume, reticulocytes, peripheral blood smear, number of leukocytes, leukocyte formula, number of thrombocytes.
\end{itemize}
\end{enumerate}

tumoral syndrome, superficial latero-cervical adenopathies, mediastinal adenopathies, abdominal adenopathies, splenomegaly, hepatomegaly, other tumors.

\begin{itemize}
\item \textbf{Initial blood count and throughout the disease:} hemoglobin, mean red blood cell volume, reticulocytes, peripheral blood smear, number of leukocytes, leukocyte formula, number of thrombocytes.
\item \textbf{Complete ENT examination, including panendoscopy:} the examination by rigid or flexible endoscopy (under local anesthesia with 2% lidocaine) of the rhino-pharynx and of the larynx, followed by the removal of a biopic fragment from the tumoral masses discovered in the rhinopharynx, at the base of the tongue. For tumoral masses of Waldeyer’s ring, only the excisional biopsy is the correct way of diagnosis. If no tumor mass is detectable, 6–8 “blind” biopsies are to be excised from the target areas.
\item \textbf{The pathology examination of the excised fragment:} imprint and paraffin examination, correlated with the histochemical examination. A lymph node biopsy is to be performed under general anesthesia with orotracheal intubation.
\item \textbf{Initial medullogram and in the course of evolution of the disease:} bone marrow smears are examined. These are obtained by puncturing the sternum or iliac crest, and are colored MGG (May–Grüwald–Giemsa); the immunohistochemical examination of the bone marrow biopsy is performed using efficient monoclonal antibodies.
\item \textbf{Biochemical parameters:} blood sugar, liver function – transaminases (GOT, GPT), \(\gamma\)-glutamyl-transpeptidase (GGT), lactate dehydrogenase (LDH), serum alkaline phosphatase (sAP), bilirubin, renal function – urea, uric acid, serum creatinine, coagulogram, serum proteins, albumin, \(\beta\)-2 microglobulin, C-reactive protein.
\item \textbf{Immunologic assessment:} bone marrow and peripheral blood immunophenotyping.
\item \textbf{Molecular biology:} PCR and RT-PCR.
\end{itemize}

The used monoclonal antibodies have targeted certain markers of differentiation and prognosis: Bcl-6, Ki67, Bcl-2, CD5, CD10, CD15, CD30, and CD20.

The patient’s chart includes a special section with the immunohistochemical profile: CD20, Bcl-2, Bcl-6, CD10, Ki67, CD20, and CD5.

The clinical risk categories in our study were based on the international prognosis index of follicular lymphoma (FLIPI) \([8]\), which comprises five negative prognostic factors:

\begin{itemize}
\item age over 60 years;
\item stage Ann Arbor III or IV;
\item hemoglobin <12 g/L;
\item more than four lymph node areas involved;
\item elevated serum LDH.
\end{itemize}

Based on the above-mentioned parameters, we can define three risk groups with respect to a 10-year survival (low – 71%, intermediary – 51% and high – 36%). However, according to some authors, FLIPI is less effective in precisely identifying the high-risk group of patients than the \textit{International Prognosis Index} (IPI) \([9, 10]\).
We have analyzed possible correlations between the presence of CD5, CD10, CD20, Bcl-2, Bcl-6, and the clinical evolution, time to treatment response, failure of treatment, new adenomegaly.

**Results**

The lymph node pieces obtained by neck dissection in ENT Department, Colțea Clinical Hospital, Bucharest, in the interval 2004–2010, have been analyzed and classified as Hodgkin and non-Hodgkin lymphomas based on the pathology examination with standard staining (Table 1, Figure 1).

**Table 1 – Number of cases distribution of lymphoma between neck dissections**

<table>
<thead>
<tr>
<th>Year</th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of neck dissections</td>
<td>156</td>
<td>207</td>
<td>202</td>
<td>228</td>
<td>248</td>
<td>256</td>
<td>253</td>
</tr>
<tr>
<td>Hodgkin lymphoma</td>
<td>64</td>
<td>45</td>
<td>54</td>
<td>63</td>
<td>71</td>
<td>55</td>
<td>64</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>28</td>
<td>14</td>
<td>31</td>
<td>33</td>
<td>41</td>
<td>36</td>
<td>30</td>
</tr>
</tbody>
</table>

A tendency of increment is observed from the point of view of the number of cases which presented to the ENT Department, the number of both Hodgkin and non-Hodgkin lymphomas increasing accordingly in the 2004–2008 interval, and a slight decrease after 2008.

**Figure 1 – Case distribution of lymphoma between neck dissections.**

For our study, we have chosen 58 patients based on pathology characters, and the results of the oncogenes determination Bcl-2 and Bcl-6.

As far as the molecular events involved in the prognosis and prediction of follicular lymphoma are concerned, they can be characterized immunohistochemically or by means of molecular genetics methods.

An attempt was made to correlate the presence of certain immunohistochemical and cytogenetic markers with the evolution and aggressiveness of the disease.

We present here the results of the analysis obtained from the cohort of 58 patients with follicular lymphoma (out of 104 patients with non-Hodgkin malignant lymphoma from this study).

The median age of the cohort is 57.78 years (standard deviation 13.104) and 41.3% patients are in the group 40–59-year-old, corresponding with the indolent nature of the follicular lymphoma. Twenty-two cases were classified as grade 1 follicular lymphoma, according to WHO classification.

The tumor characters at the time of diagnosis: 25 patients had superficial nodes <5 cm, 10 patients had superficial and deep nodes <5 cm, but six patients presented with bulky disease.

The majority of patients of the cohort were diagnosed based on neck dissection, because 50 cases had adenomegaly, while eight patients had specific ENT localization: Waldeyer’s ring two cases, paranasal sinus two cases, rhinopharynx four cases, salivary gland one case, and thyroid gland tumor one case (Figure 2).

**Figure 2 – The site of the biopsy for the diagnosis in the cohort of follicular lymphoma.**

We present the results of the CD10, CD5 and CD20 analysis for the differential diagnosis of lymphoma, and also for the correlation with the evolution. CD10 was positive at 30 patients – 51.7% (Figure 3).

**Figure 3 – The expression of CD10 in follicular lymphoma.**

CD5 was negative in 96.6% of the cases, specific for the histology type of follicular lymphoma.

CD20 was positive at 45 patients, meaning 77.6%, important for the decision of treatment with anti-CD20 antibody.

We also studied the expression of proteins corresponding to oncogenes, Bcl-2 and Bcl-6, and we obtained the results presented as follows: Bcl-2 was positive in 50% of the cases and negative in eight cases (Figure 4). The ratio of Bcl-2 positive lymphoma is less in follicular lymphoma grade 3 and is raised in grade 1 follicular lymphoma.

Bcl-6 was determined in 55.2% of the cases, and it was positive in 12 cases (20.7%) and negative in 20 cases (34.5%) (Figure 5).
Figure 4 – Overexpression of Bcl-2 in follicular lymphoma.

Figure 5 – Overexpression of Bcl-6 in follicular lymphoma.

At the end of the study, after the treatment, we obtained 28 total remissions (48.3%), seven patients with partial remission and seven patients with progressive disease, and 16 patients deceased (Figure 6).

Figure 6 – Clinical evolution at the end of the study.

The results obtained after the Hematoxylin–Eosin staining pathology examination, as well as the immuno-histochemical data obtained in cooperation with the “Victor Babeș” National Institute, Bucharest, are subsequently presented (selection of cases) (Figures 7–12).

Figure 7 – Non-Hodgkin malignant lymphoma (NHML): nodular pattern, small B-cells. Bcl-2 diffuse positive reaction (ob. ×20).

Figure 8 – NHML: small cells mantle type & nodular pattern. CD23 positive in germinal centre (ob. ×10).

Figure 9 – NHML: follicular diffuse pattern. CD68 stain. Positive perinodular macrophages (ob. ×10).

Figure 10 – NHML: follicular diffuse pattern. Ki67 stain. Proliferation index 7–8% (ob. ×40).
Discussion

Patients with lymphomas from 2004 through 2010 have been identified and a database has been formed as a starting point for the study, and we can observe an increase of the cases of lymphoma.

The unitary diagnosis and staging protocol has been established, taking into account the new data available in literature and we can say that the clinical classification and the initial pathology diagnosis of the cases allow a good orientation regarding the immunologic testing.

Most of the cases were diagnosed in stages 3 or 4, corresponding to the data from the literature and according to indolent evolution of this type of lymphoma.

The difficulties in the pathology diagnosis occur especially in the cases with a predominance of centroblastic type large cells.

Most patients with follicular lymphomas present with the t(14;18)(q32;q21) translocation (which is encountered in 85–90% of cases). This causes the juxtaposition of the (JH) segment of the heavy immunoglobulin chain locus from the 14th chromosome and of the Bcl-2 proto-oncogene from the 18th chromosome, resulting in the inhibition of apoptosis and the prolonged survival of the cell, thus promoting the occurrence of successive mutations, which determine the malignant phenotype [11–14].

Most of the patients with follicular lymphomas present with circulating cells that bear the Bcl-2 rearrangement, even in incipient stages of disease, thus offering the unique opportunity of identifying and classifying them without necessarily requiring the direct analysis of the tumoral tissue. The degree of concordance between the data obtained by analyzing the peripheral blood or bone marrow and that obtained by analyzing the tumoral tissue is of 95%, corresponding to literature data [15–17].

Our correlation between evolution and oncogene protein presence shows that the Bcl-2 overexpression might be a marker of poor prognosis [18], whereas the expression of Bcl-6 or CD10 (germinative center markers) indicated a favorable prognosis [19, 20].

We found that Bcl-6 is positive especially in the tumor forms associated with the predominance of small cells, likewise other studies we found on follicular lymphoma [21, 22].

Anti-CD20 therapy became the rule [23] but there are tumors, which are not responding, or tumors that became resistant at the therapy, probably developing complex resistance mechanisms.

The most important thing might be that patients with the same diagnosis and a similar treatment may present with significant differences regarding the clinical manifestations, the molecular profile and the evolution [24–26].

Conclusions

All tested tumors have proven positive for CD20, which is an essential marker for targeted immunotherapy.

The CD10 expression is more frequent and more intense in well-differentiated non-Hodgkin lymphoma, follicular type.

The Ki67 proliferation index shows superior values in the NHL follicular type with the predominance of centroblastic-type cells and can be related with the aggressiveness of the tumor, corresponding with data found in literature at similar studies.

Bcl-2 is positive in all tumor subtypes, being associated relatively frequently with CD5 expression, and is a marker of poor prognosis.

Bcl-6 is positive especially in the tumor forms associated with the predominance of small cells and is a marker of favorable prognosis.

The identification of markers of transformation in highly malignant lymphomas is necessary.

Monitoring the therapy in non-Hodgkin malignant lymphomas (NHML) is a difficult task due to the heterogeneity of tumoral subtypes.

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