Individual risk prediction of nodal and distant metastasis for patients with typical bronchial carcinoid tumors

Joao-Carlos Das-Neves-Pereira, Patrick Bagan, Jose-Ribas Milanez-de-Campos, Vera-Luiza Capelozzi, Claire Danel, Fabio-Biscegli Jatene, Jean-Francois Bernaudin, Marc Riquet

Abstract

Objective: Bronchial typical carcinoid tumors are low-grade malignancies. However, metastases are diagnosed in some patients. Predicting the individual risk of these metastases to determine patients eligible for a radical lymphadenectomy and patients to be followed-up because of distant metastasis risk is relevant. Our objective was to screen for predictive criteria of bronchial typical carcinoid tumor aggressiveness based on a logistic regression model using clinical, pathological and biomolecular data. Methods: A multicenter retrospective cohort study, including 330 consecutive patients operated on for bronchial typical carcinoid tumors and followed-up during a period more than 10 years in two university hospitals was performed. Selected data to predict the individual risk for both nodal and distant metastasis were: age, gender, TNM staging, tumor diameter and location (central/peripheral), tumor immunostaining index of p53 and Ki67, Bcl2 and the extracellular density of neoformed microvessels and of collagen/elastic extracellular fibers. Results: Nodal and distant metastasis incidence was 11% and 5%, respectively. Univariate analysis identified all the studied biomarkers as related to nodal metastasis. Multivariate analysis identified a predictive variable for the individual risk of these metastases to determine patients eligible for a radical lymphadenectomy and patients to be followed-up because of distant metastasis risk. Conclusions: Individual risk prediction of bronchial typical carcinoid tumor metastasis for patients operated on can be calculated in function of biomolecular data. Prediction models could detect high-risk patients and help surgeons to identify patients requiring radical lymphadenectomy and help oncologists to identify those as having an aggressive disease requiring prolonged follow-up.

Keywords: Carcinoid tumors; Neuroendocrine carcinoma; Metastasis; Prediction; Logistic regression; Angiogenesis

1. Introduction

Bronchopulmonary carcinoid tumors are neuroendocrine proliferations, commonly classified in two groups: typical and atypical [1]. Typical carcinoid tumors are considered as a non-metastatic disease. Surgery is the treatment of choice of bronchopulmonary typical carcinoid tumors (BTCT), but the extension of lung resection and the need of lymph node dissection remain controversial [2,3]. Some surgeons do not perform radical lymph node dissection, while others advocate that it must be performed [4]. Some authors believe that it can be a therapeutic procedure, others believe that it is only a diagnostic procedure in order to achieve a better nodal staging [2]. Identifying variables related to the subgroup of BTCT with lymph node metastatic potential would assist prediction of the individual risk of nodal metastasis and help surgeons’ decisions for each individual patient [5,6]. Distant metastasis also must be predicted as the postoperative follow-up of an aggressive disease requires screening of metastasis that includes image exams and blood tests. Epidemiological, clinical, pathological
and biomolecular features have already been described as being related to the BTCT metastatic potential: for example among clinico-pathological characteristics: male gender, elder age, peripheral location, and a larger diameter or among cellular biomarkers a high nuclear immunexpression of p53 or proliferation markers as Ki67 and a low expression of apoptotic markers (Bcl2, Bax, caspases, Fas, Fas-ligand). Among components of the stroma the following characteristics have been hypothesized to be associated with a more aggressive behavior: a low density of extracellular matrix collagen and elastic fibers, an immunostaining by anti-heparanase antibodies and the presence of an intense neo angiogenesis (stained by anti-CD34 antibodies) [7—9]. Mathematical models are used in medicine to predict morbid events, as logistic regression, artificial neural network and genetic algorithm [10]. In order to predict the individual risk of nodal and distant metastasis in patients with BTCT, clinical, pathological and biomolecular variables were analyzed under the logistic regression predictive model.

2. Patients and methods

A multicenter retrospective cohort study, including 202 French and 128 Brazilian consecutive patients operated on for BTCT and followed-up during a period longer than 10 years in two university hospitals was performed.

2.1. Diagnosis confirmation

All the resected pathological specimens of the primary tumor were reviewed and reclassified by the same two pathologists who analyzed all the specimens together; both of them with a great experience in pulmonary neuroendocrine carcinomas. Only patients whose pathological review confirmed typical carcinoid tumor, according to the 2004 WHO classification [1], were included in the study.

2.2. Inclusion criteria

Patients with nodal metastasis (all of them confirmed by pathological revision) were included in the group of patients with nodal metastasis.

Patients without any metastasis who were followed-up for more than 10 years were invited to a physical examination and a CT scan including chest, brain, liver and suprarenal images. Normal mediastinal image exams were required to include patients in the group without nodal metastasis.

Patients without any metastasis who were followed-up for more than 10 years were invited to a physical examination and a CT scan including chest, brain, liver and suprarenal images. Normal mediastinal image exams were required to include patients in the group without nodal metastasis. Patients with abnormal mediastinal images underwent diagnostic investigation by mediastinoscopic biopsies. Those with mediastinoscopy biopsy confirming nodal metastasis were included in the group with nodal metastasis.

Patients with abnormal physical examination or with abnormal brain, liver or suprarenal images should be submitted to additional diagnostic procedures.

2.3. Exclusion criteria

Patients without any metastasis who were not followed-up for more than 10 years and who were not volunteers to be examined were not included in the study.

2.4. Clinical and pathological data

Medical charts were reviewed and the following clinical and pathological data were collected: age at the moment of operation, gender, ethnic group, preoperative weight loss and chest pain, TNM staging, tumor diameter and volume, and location (central/peripheral).

2.5. Immunohistochemistry

Tumor tissue samples were obtained during surgical treatment of BTCT and fixed in 10% formalin. For each case, one or two slides of the primary tumor were selected by light microscopy. Acceptable sections were those that represented the predominant histological subtype identified on the majority of slides, with at least 10 microscopic fields at a magnification of X250 representing BTCT and minimal preparation artifact. Their respective paraffin-embedded blocks were sectioned at 3 μm and stained with H&E. Two pathologists reviewed these slides separately in a randomized fashion and agreed on the diagnosis of typical carcinoid. Travis’ diagnostic criteria for BTCT were applied and all specimens showed less than 2 mitoses/10 high power fields. Mitoses rates were calculated using an Olympus microscope, where the field of view was 0.2 mm², so 10 high power fields equaled 2 mm².

2.6. Tissue biomarkers

2.6.1. Nuclear p53 and Ki-67 and cytoplasm Bcl2 staining

The presence of p53, Ki-67, and Bcl2 was analyzed by immunohistochemical (IHC) staining on formalin-fixed, paraffin-embedded tissue using the avidin–biotin peroxidase complex technique. For each case, paraffin blocks that presented minimal preparation artifact were used for morphologic and morphometric analysis. Histological sections were cut at 3 μm, mounted onto poly-L-lysine-coated slides, and incubated overnight at 4°C with the primary antibody. The antibodies used were Bcl2 mouse monoclonal clone 56-2A4 at a dilution of 1:100 (Biogen), monoclonal mouse anti-human p53 protein (DO7; Dako A/S, Glostrup, Denmark; dilution 1:40), anti-Ki-67 antigen (Dako A/S, Glostrup, Denmark, dilution 1:1800).

Brownish nuclear staining was considered as evidence of antigen expression by cells according to nuclear Ki-67, p53, and cytoplasm staining for Bcl2 antibodies. In order to determine the index of expression for each antibody, it was used to judge the intensity ("hot spots") and the percentage of labeled cells at a magnification of 400×, counting a total of 1000 cells that covered an area of 62,500 μm²/field/section, actually representing the density of stained positive cells in areas of tumor tissue. For measurements, a standard microscope was equipped with an eyepiece (numerical aperture, X10) containing a reticulated grid with 100 points and 50 lines. The immunohistochemistry indices of expression (IIE) were obtained by the following relationship:

\[
IIE = \frac{Pih}{Pt} = (\% \ section)
\]
where \( P_{ih} \) and \( P_{t} \) represent the numbers of points overlying tissue-stained cells and tumor, respectively, expressed as a percentage.

2.7. Extracellular stromal biomarkers

2.7.1. Pathological neoformed microvessel density

The microvessel density (MVD) was evaluated using anti-CD34 monoclonal antibody (Novocastra Laboratory, Newcaste, United Kingdom) at a 1:25 dilution, applying the same IHC procedure as for p53, Ki-67, and Bcl2. Microvessel quantification was evaluated in the region of highest vessel density. For each slide, the 10 most vascular areas within the tumor mass were chosen. At X200, the field in each of these 10 areas was counted by conventional point counting, and the average counts of the 10 fields were recorded. A vessel lumen was not required for the identification of a microvessel; single endothelial cells or cell clusters were counted. Large vessels with thick muscular walls or with lumina greater than 50 \( \mu m \) were excluded from the count.

2.8. Collagen—elastic extracellular matrix components

Collagen was stained in formalin-fixed, paraffin-embedded tissue sections (5 \( \mu m \) thick), stained in a 0.2% solution of Sirius red (Direct Red 80, C.I. 35780, Aldrich, Milwaukee, WI), and dissolved in aqueous saturated picric acid. Since 1964, this dye has been used for staining collagen in histological specimens and was demonstrated to allow quantitative analysis of collagen also in paraffin sections. The enhancement of collagen birefringence promoted by the picrosirius polarization method is specific for collagenous structures composed of aggregates of orientated molecule.

Elastic system fibers stain was performed by applying Weigert’s resorcin—fuchsin method, modified with a previous oxidation. This method stains the three types of elastic system fibers (elastin, oxytalan and fully developed elastic fibers) with the possibility of quantifying all the elastic tissue in the sample, without losing the oxytalan component.

Different fields were randomly selected, and the density of total ECM, as well as that of collagen and elastic fibers, was determined by optical density in the image analysis system using Bioscan-Optimas 5.1 software (Bioscan, Inc; Edmonds, Wash). A total of 10 fields per case were analyzed at a magnification of \( \times 400 \). The threshold for collagen and elastic fibers was selected for individual cases after enhancing the contrast up to a point at which the fibers were easily identified as black (elastic) or white (collagen) strips. The area occupied by the fibers was determined by digital densitometry recognition, and the results expressed the amount of collagen system and elastic system fibers (in area) per ECM unity. The amount of collagen system fibers added to the amount of elastic system fibers represents total ECM density.

2.9. Statistical analysis

SPSS statistical program (SPSS Inc., Chicago, IL, USA) was used. Logistic regression was chosen as a predictive model.

A forward stepwise conditional method was applied. The criterion for choosing the final model was the last step.

Independent variables included in the logistic model as candidates to predict nodal and distant metastasis were: gender, age in years, central/peripheral position, positive microscopic edge resection for malignant cells, larger diameter in mm, p53, Ki67 and Bcl2 staining index, neoformed pathological microvessels density and extracellular fibers of the collagen and elastic system density.

Gender was included as a categorical variable and central/peripheral location as a dichotomous variable.

Outliers were included in the model, but each case was also analyzed separately.

3. Results

Lymph node metastasis incidence was 11% and 10% for Brazilian and French patients respectively. In all patients, except one, nodal metastases were diagnosed at the pre- or intra-operative moment.

Demographic and pathological data were recovered from medical charts. There were 184 female and 146 male patients, the mean and standard deviation of age was 46.8 \( \pm \) 0.5 years old, ranging from 1 to 83. There were 238 central and 92 peripheral tumors, the mean and standard deviation of tumor diameter was 27.7 \( \pm \) 16.3 mm, ranging from 5 to 80 mm.

All the studied biomarkers were related to nodal metastasis in the univariate analysis (Table 1). Male gender was borderline related to the phenomenon, but it was not statistically significant. Multivariate analysis showed that predictive independent variable for lymph node metastasis was angiogenesis, (microvessels density: CD34 immunostained endothelium). The beta coefficient was \( \beta = 0.3 \), exp \( \beta = 1.378 \) (CI 95%: 1.153—1.647 Tables 2 and 3) with a standard error = 0.1 (\( p = 0.0001 \)). The logistic equation had a constant = −5.2; with a standard error = 1.3 (\( p = 0.0001 \)).

Distant metastases incidence was 5% in both French and Brazilian cohorts.

Although univariated analysis identified the male gender as related to distant metastases, logistic regression did not identify it as a predictive variable (\( p = 0.058 \), Table 3).

Three outlier patients who were not predicted but had distant metastasis were analyzed separately and their cases were published previously.

4. Discussion

Our study does not support that all the tumors classified as BPTC belong to the same pathological entity. We suggest that

### Table 1

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Nodal metastasis</th>
<th>( p^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without metastasis</td>
<td>With metastasis</td>
</tr>
<tr>
<td>Ki-67</td>
<td>1.9 ± 2.1</td>
<td>4.2 ± 3.0</td>
</tr>
<tr>
<td>p53</td>
<td>1.9 ± 2.1</td>
<td>3.9 ± 2.7</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>9.7 ± 4.6</td>
<td>5.0 ± 4.4</td>
</tr>
<tr>
<td>CD-34</td>
<td>6.9 ± 4.0</td>
<td>16.1 ± 5.9</td>
</tr>
<tr>
<td>Collagen fibers</td>
<td>12.1 ± 6.3</td>
<td>5.8 ± 5.9</td>
</tr>
<tr>
<td>Elastic fibers</td>
<td>14.3 ± 4.1</td>
<td>8.4 ± 5.9</td>
</tr>
</tbody>
</table>

* Student’s t-test.
and really aggressive metastatic carcinomas [11—13].

Hyperplasia, including adenomas, low-grade carcinomas and differentiated neoplasias [14,15]. Some patients had a peripheral well vascularized tumors. Angiogenesis seems to be related to a worse prognosis in other types of well-differentiated neoplasias [14,15]. Some patients had a several year clinical history of pneumonia in the lobe harboring the tumor, corroborating the hypothesis that in these special cases, the tumors were benign. One patient with a histologically proven diagnosis of carcinoid tumor refused surgery during 7 years: when he was operated on, there was neither adjacent tissues invasion nor metastasis and he remained disease free 10 years during the postoperative follow-up. On the contrary, tumors may demonstrate a very aggressive biological behavior. A patient with a 5 mm diameter tumor, classified as tumorlets had nodal metastasis. Some African American females from the Caribbean area had tumors following the bronchial tree in a ramifying pattern, histologically without mitosis figure or any necrosis area, but they died because of metastases [16]. All these observations demonstrated that we are probably facing several different pathological entities, which however are considered as typical carcinoid tumors.

Surgical treatment and long-term follow-up are reflecting this controversial subject [17], raising the question whether patients with tumors without metastatic behavior and those with tumors harboring a real aggressive metastatic behavior should be treated in the same way and be followed-up during the same period with the same exams [18].

The extension of surgical pulmonary parenchyma resection and lymph node dissection to treat BTCT must consider the individual risk of nodal metastasis. Specific postoperative follow-up and future adjuvant therapies indication must consider the individual risk of distant metastasis [14,19].

The current WHO criteria are not sufficient: they identify only metastatic tumors as potentially atypical carcinoid tumors by analyzing the primary tumor [20]. Necrosis and mitoses are criteria with a good positive predictive value in order to predict prognosis, but they do not have a good negative predictive value. Even patients with the entire tumor without any necrotic area and without any mitosis figure can send metastasis. Surgeons must know the actual risk of metastasis in the preoperative period to determine the extension of parenchyma and nodal resection to perform at surgery.

The advantage of using immunohistochemical biomarkers as a preoperative predictive tool is that a small fragment of tumor is sufficient to evaluate their potential aggressiveness [21,22], while WHO criteria require the analysis of the whole tumor, only obtained postoperatively. It must be underlined that the majority of BTCT are endobronchial lesions and biopsies can be done by fiberoptic bronchoscopy and moreover some peripheral tumors can be reached by transthoracic needle biopsies [23]. Some authors do not perform endobronchial biopsies of tumors with macroscopic features of typical carcinoid tumors, due to the risk of bleeding [24]. In our study 219 patients (66%) had a preoperative bronchoscopic biopsy. In 43 of these 219 biopsies (12.6%) there was a degree of bleeding. In 19 of these 43 cases of bleeding (44% of bleeding biopsies and 8.6% of the total 219 biopsies) it was necessary to use hemostatic bronchoscopic procedures as instillation of cold crystalloid fluids with adrenaline. All these bleedings were successfully controlled with these bronchoscopic procedures.

Our set of markers assists prediction of the usefulness of performing lymph node resection while it does not assist prediction of distant metastasis. However, positive lymph nodes were demonstrated to have a tendency to be related to distant metastasis. In the present series the too small number of cases of distant metastasis did not allow identifying predictive variables needing the inclusion of more patients.

Predicting distant metastasis also presents several practical applications regarding postoperative follow-up and future adjuvant therapies. Octreoscan is a specific image exam that could be performed in the follow-up of high-risk patients [18]. The dosage of the level of chromogranin-A in blood tests has been already described as a metastasis-screening tool in carcinoid tumors [25]. In the future, targeted therapies as radio isotopes (Lutetium) guided by somatostatin analogues against metastatic neuroendocrine cells could be offered to high-risk selected patients [19].

In conclusion, these results demonstrate a practical implication. Individual risk prediction of nodal metastasis allows surgeons to tailor a personalized treatment to each patient. Unnecessary radical operations in low-risk patients as well as leaving positive nodal metastasis in high-risk patients may be avoided.

These are only initial results, and only part of a large group of study that is still trying to find more predictive biomarkers and more predictive models. So, prediction will be more precise when:

1. Larger cohorts will be followed-up: our group is a multinational multicenter group. The present article includes only some of the Brazilian and French patients, those who were already metastatic or not with a follow-up after the tenth postoperative year. In this report we do not include either a Portuguese cohort that

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**Table 2**

Predictive variables of nodal metastasis in typical bronchopulmonary carcinoid tumors (logistic regression multivariate analysis)

<table>
<thead>
<tr>
<th>Predictive variable</th>
<th>β</th>
<th>p</th>
<th>Exp (β)</th>
<th>95% CI Exp (β)</th>
<th>Lower</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34</td>
<td>0.321</td>
<td>0.0001</td>
<td>1.378</td>
<td>1.153 — 1.647</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constant</td>
<td>−5.244</td>
<td>0.0001</td>
<td>0.005</td>
<td></td>
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</table>

95% CI: confidence interval 95%.

**Table 3**

There was no predictive variable of distant metastasis in typical bronchopulmonary carcinoid tumors (logistic regression multivariate analysis)

<table>
<thead>
<tr>
<th>Predictive variable</th>
<th>β</th>
<th>p</th>
<th>Exp (β)</th>
<th>95% CI Exp (β)</th>
<th>Lower</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender</td>
<td>2.153</td>
<td>0.058</td>
<td>8.610</td>
<td>0.931 — 79.608</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constant</td>
<td>−3.434</td>
<td>0.001</td>
<td>0.032</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

95% CI: confidence interval 95%.
was recently recruited, nor Brazilian and French patients without metastasis who were followed-up for less than 10 years. But each year more and more Brazilian, French and Portuguese patients will be included in the study because they will complete a 10 year follow-up, and other groups have also been invited to integrate this effort.

2. An outpatient nucleus specialized in bronchopulmonary carcinoid tumor was established in our service in Sao Paulo University, Brazil. We are planning to follow this model in our French and Portuguese hospitals. Specialized outpatient nucleus makes easier to keep on contact with treated patients.

3. New predictive epidemiologic, clinical, pathological variables and new biomarkers will be discovered. Prospective storage of frozen tissues is being performed and will allow the tumor mRNA study; transcriptome data could be evaluated.

4. More powerful predictive models will be applied, as artificial neural network and genetic algorithm.

References


Appendix A. Conference discussion

Dr E.A. Rendina (Rome, Italy): Dr Neves-Pereira and his colleagues should be congratulated for carrying out an interesting review of their experience on bronchial carcinoids with the specific aim of providing reliable information on the risk prediction of nodal metastasis. I have to note, however, that the abstract I received was, unfortunately, quite different from the actual presentation. I hope the final manuscript will clarify these discrepancies. Because I was not able to read the paper in advance, I will limit myself to a brief comment and a few questions.

Like most retrospective multicenter studies on carcinoids, the present one, though based on a very large number of patients, suffers from the possible heterogeneity of treatment between the centers, the various centers, and the long timespan of the review. Notwithstanding, I think that the main result of the study, namely that the occurrence of metastases is dependent from the degree of neoangiogenesis, seems quite independent from the treatment strategy. This information can therefore be seen as a remarkable contribution in this field.

My questions are the following:

Was actually all the histological material reviewed by the same team of pathologists?

And secondly, are there any surgical and/or therapeutic implications to your study?

Dr Neves-Pereira: First of all, when I was preparing this presentation, I would like to present the risk of metastasis of typical and atypical carcinoid tumors and also the risk of distant metastasis. It would be a very extensive presentation, so I sent to the Congress the abstract about typical carcinoid tumors because I believe that this is the real problem.

But for the presentation, when I included the distant metastasis results and its discussion, it was too long to be presented in 8 minutes, so I have chosen to show only the nodal metastasis. I consider that, as we are thoracic surgeons, we can resect only the nodal metastasis in the operative act, but we cannot resect the distant metastasis. Concerning these distant metastasis, perhaps oncologists will follow these patients and perhaps offer them complementary surgical abdominal operation, or some other kind of adjuvant treatment.
The second answer, we have checked 2 kinds of tissue specimens. First of all, we have checked the primary tumor because, as these patients were classified based on their medical chart as having typical carcinoids, the number of mitoses (a criteria to classify them as having typical or atypical carcinoid tumors) has changed from 5 to 2 nowadays. And so some patients who were considered as having typical carcinoids, they had in fact, for example, 3 mitosis figures in the microscopic analysis.

Dr Rendina: I’m sorry to interrupt you. Probably my question was not clear. My question was very precise. Did the same team of pathologists review all the material?

Dr Neves-Pereira: Yes, we have taken the hematoxylin-eosin stained tissues, and we analyzed them.

Dr Rendina: And the second question was: Do you see any potential surgical or therapeutic implication to this study?

Dr Neves-Pereira: Yes. In Brazil we are used to follow-up all the patients with typical carcinoids. And now, as we can collect a small fragment, for example, by bronchoscopic biopsy from central tumor, we can analyze these biomarkers preoperatively and so we can perform lymph nodal resection routinely in these patients. Perhaps this does not change anything in some services (those that resect lymph nodes routinely in these patients).

Dr W. Klepetko (Vienna, Austria): In your opening statement you mentioned that it is a mixture of retrospective and prospective work. Could you once more elaborate, what was the retrospective part of the work and what will be the prospective part.

And in addition, I would like to ask you about the surgical procedures performed, about the uniformity. It you look for micrometastases in nodes, it’s very important that the operative therapy is standardized with regard to lymph node sampling. Could you give us some information on how that was handled in the different institutions in a standardized way.

Dr Neves-Pereira: In the prospective study we collect fresh tissues, in order to perform microarray, so they must not be completely stored in paraffin-embedded blocks. So we are making prospective studies in order to perform microarray studies. But in this presentation, I present only the retrospective branch of the study, in which we analyzed only the embedded paraffin blocks.

And the second question?

Dr Klepetko: The standard surgical procedure.

Dr Neves-Pereira: About standard surgical procedure, we had some problems with some patients, the first patients, for example, in the 1980s. So for this reason we followed them for 10 years and we have analyzed their CT scan, because we have to be sure that they didn’t have progression with mediastinal image.