A COMPARISON OF NSP-RETICULONS WITH CONVENTIONAL NEUROENDOCRINE MARKERS IN IMMUNOPHENOTYPING OF LUNG CANCERS

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SUMMARY

Neuroendocrine-specific protein (NSP)-reticulons are endoplasmic reticulum-associated protein complexes, which have been identified as markers for neuroendocrine differentiation. In this study, the expression of two members of the family of NSP-reticulons, NSP-A and NSP-C, have been investigated in different types of lung cancer and compared with the expression patterns of five conventional neuroendocrine markers, the neural cell adhesion molecule (NCAM), synaptophysin, chromogranin A, Leu-7, and neurofilament proteins. NSP-A and NSP-C antibodies were reactive with most carcinoid tumour and small cell lung carcinoma (SCLC) cases, while atypical carcinoid tumours showed a variable expression. In the total group of neuroendocrine tumours, a high concordance of expression was found between NSP-A and NSP-C, while their expression correlated well with NCAM and synaptophysin positivity. Chromogranin A, Leu-7, and neurofilament proteins were shown to be expressed to a limited extent in these neuroendocrine tumours. In a selected group of non-SCLCs known to exhibit neuroendocrine features, NSP-A expression was detected at much higher frequency than NSP-C. In virtually all NSP-A positive cases, this expression was associated with one or more of the other neuroendocrine markers. NSP-A expression showed a stronger correlation with conventional neuroendocrine markers than NCAM. In detecting neuroendocrine differentiation in non-SCLC, NSP-A is more sensitive than synaptophysin, chromogranin A, Leu-7, and neurofilament proteins. It is concluded that NSP-reticulons are valuable markers in the diagnosis of neuroendocrine differentiation in non-SCLC and should be used in conjunction with NCAM. © 1997 by John Wiley & Sons, Ltd.

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KEY WORDS—NSP-reticulons; NCAM; synaptophysin; chromogranin A; neurofilament proteins

INTRODUCTION

Neuroendocrine-specific protein (NSP)-reticulons have been described as indicators of neural and neuroendocrine differentiation in normal and malignant cells.1–3 In the majority of neuroendocrine tissues, these endoplasmic reticulum-associated aggregates consist of two closely related protein constituents, NSP-A and NSP-C. In lung cancer diagnosis, NSP-A appeared to be a reliable marker for the detection of neuroendocrine differentiation, since most of the small cell lung carcinomas (SCLCs) and all carcinoid tumours examined showed expression of NSP-A. In general, typical non-SCLC showed no expression of these markers.2 Interestingly, however, NSP-A expression was also detected in a subset of non-SCLC, in association with expression of other neuroendocrine markers, the neural cell adhesion molecule (NCAM) and/or neurofilament proteins.2 Also, in the cell lines derived from these lung cancer subtypes, expression of both NSP-A and NSP-C was found in SCLC cultures and not in typical non-SCLC cell lines.2 The reaction patterns observed in the group of histologically heterogeneous non-SCLCs are also reflected in morphologically non-SCLC cell cultures with neuroendocrine characteristics (non-SCLC-NE).4 In such lung cancer cell lines, NSP-A and NSP-C are differentially expressed.3,4 Since NSP-A and NSP-C expression was found not to depend on neurosecretory granule density, they are considered to be more sensitive markers than synaptophysin and chromogranin A.3,4

In the present study, NSP-A and NSP-C expression was investigated in a series of bronchopulmonary neoplasms, comprising carcinoids, SCLCs, and non-SCLCs. Their value as neuroendocrine markers was assessed by a comparison with other indicators of neuroendocrine differentiation, namely NCAM, synaptophysin, chromogranin A, Leu-7, and neurofilament proteins. In particular, the detection of neuroendocrine differentiation in non-SCLC may be of prognostic relevance, since evidence exists that lung tumours with such a phenotype are chemosensitive.5

MATERIALS AND METHODS

Tumour specimens

Surgical specimens of 88 cases of bronchopulmonary tumours were obtained from the Department of...
Pathology, St. Antonius Hospital, Nieuwegein, The Netherlands. All tumours were rapidly frozen, stored in liquid nitrogen, and classified on the basis of routine histological haematoxylin and eosin staining criteria, according to the latest WHO classification. No subdivision was made into poorly, intermediate, and well-differentiated non-SCLCs.

Antibodies

The following primary antibodies were used in this study:

(a) Mouse monoclonal antibodies RNL-2 and RNL-3 (both IgG1 subtype), recognizing epitopes present in NSP-A, were used as undiluted culture supernatants.

(b) Mouse monoclonal antibodies MON-160, MON-161, and MON-162 (all of the IgG1 subtype) specific for NSP-A were used as a 1:1:1 mix (MON 160–162) in a 1:5 dilution of culture supernatant.

(c) Polyclonal rabbit antiserum POL-1, recognizing epitopes present in NSP-A, was used in a 1:2500 dilution.

(d) Mouse monoclonal antibody RNL-4 (IgG1 subtype) specific for NSP-C was used in a 1:4 dilution of culture supernatant.

(e) Mouse monoclonal antibody RNL-1 (IgG1), recognizing NCAM, was used as undiluted culture supernatant.

(f) Polyclonal rabbit antiserum to synaptophysin (DAKO A/S, Glostrup/Denmark), a component of synaptic vesicles and widely distributed in neurons and neuroendocrine cells, was used in a 1:50 dilution.

(g) Mouse monoclonal antibody LK2H10, recognizing chromogranin A (IgG1 subtype; BioGenex, San Ramon, CA, U.S.A.), was diluted 1:100. Chromogranin A is associated with dense core vesicles and is present in neural and neuroendocrine cells.

(h) Mouse monoclonal antibody HNK-1 (IgM), recognizing Leu-7 (Becton Dickinson, Mountain View, CA, U.S.A.), was diluted 1:10.

(i) Mouse monoclonal antibody KP1 (IgG1 subtype), recognizing CD68 on human macrophages (DAKO A/S), was diluted 1:500.

(j) Mouse monoclonal antibody MNF (IgG1), reactive with the 68 kD and 200 kD neurofilament subunits, was diluted 1:10.

(k) Polyclonal rabbit antiserum pNF68, reactive with the 68 kD neurofilament subunit, was diluted 1:400.

(l) Polyclonal rabbit antiserum pNF160, reactive with the 160 kD neurofilament subunit, was diluted 1:600.

Immunohistochemical procedure

Immunohistochemical staining was performed by the indirect immunoperoxidase technique as described before. Briefly, frozen sections (4–6 μm thick) were cut on a cryostat (cryocut 1800, Leica), air-dried, and fixed in acetone (−20°C) for 10 min. The sections were incubated with the primary antibodies for 1 h in the appropriate dilutions in phosphate-buffered saline (PBS; 0.15 M NaCl, 10 mM Na-phosphate, pH 7.2). After repeated washing in PBS, sections were incubated for 1 h with peroxidase-conjugated rabbit anti-mouse IgG (DAKO A/S), or with peroxidase-conjugated swine antirabbit IgG (DAKO A/S), both diluted 1:100 in PBS containing 5 per cent human AB serum. After washing in PBS, peroxidase activity was detected with 3-amino-9-ethylcarbazole (AEC; Sigma, St. Louis, MO, U.S.A.), was diluted 1:10.

Evaluation of immunohistochemical staining results

The different NSP-A antibodies showed virtually identical staining patterns in all cases examined. However, the monoclonal antibodies RNL-2 and RNL-3 showed in general a less intense immunoreactivity compared with the monoclonal antibody mixture MON 160–162 and the polyclonal antiserum POL-1. Since NSP-A antibodies occasionally show immunoreactivity with macrophages, those tumours in which NSP-A antibody reactivity was not confined to the tumour cells and which were also positive for CD68 were scored negative for NSP-A. In general, when virtually all tumour cells expressed a certain marker, its expression was defined as being diffuse. Focal positivity is defined as reactivity (>10 per cent) in part of the tumour, or when staining is found in scattered cells.

RESULTS

The results of the immunohistochemical reactions are summarized in Tables I–IV and illustrated in Figs 1 and 2.

Carcinoids

The immunophenotypes of the individual carcinoid cases are shown in Table II. NSP-A expression was observed in all five carcinoid cases tested. Strong cytoplasmic immunoreactivity was observed in four cases (Fig. 1A), in virtually all tumour cells, while in one case focal expression was seen. For NSP-C, a focal immunoreactivity pattern could be detected in four of the five carcinoid tumours (Fig. 1B), while one case was negative. In all these cases, NCAM expression was present as membranous staining in most cells. The two cases tested for synaptophysin were diffusely positive for this
Fig. 1—Immunoreactivity patterns of a lung carcinoid (A, B), an atypical carcinoid of the lung (C, D), and a SCLC (E–H) characterized by MON 160–162 for NSP-A (A, C, E, G), and RNL-4 for NSP-C (B, D, F, H). The bar indicates 25 μm in A–D and 45 μm in E–H.
marker; one also showed a diffuse positive reaction for chromogranin A and the other exhibited focal chromogranin A expression. Neurofilament proteins were observed in one of the three carcinoid tumours tested.

In atypical carcinoid tumours, strong diffuse NSP-A expression was observed in two out of five cases (Fig. 1C), while NSP-C was focally present in one case (Fig. 1D), which also expressed NSP-A. In these NSP-positive tumours, other neuroendocrine markers were also present: NCAM and synaptophysin were found in both NSP-positive cases, while chromogranin A and Leu-7 were found in one of these two cases. In addition, one atypical carcinoid tumour, negative for NSP, exhibited NCAM and synaptophysin. Neurofilament protein expression could not be detected in these tumours.

**SCLC**

Table II summarizes the immunophenotypes of the individual SCLC cases. The NSP-A antibodies reacted positively in six of eight cases of SCLC examined. In three cases, a focal staining reaction was seen (Fig. 1E), while in the other three positive cases diffuse immunoreactivity was present (Fig. 1G). Likewise, NSP-C could be detected in six SCLC cases examined. In two cases, diffuse expression of NSP-C in all tumour cells was...
observed (Fig. 1F) and in three cases a combined pattern was seen, in that part of the tumour was diffusely positive, while the other part showed only focal expression of NSP-C. One SCLC case was only focally positive (Fig. 1H). Of eight SCLCs, five showed co-expression of both NSP-A and NSP-C, although the intensity of expression varied for individual tumours and markers. In all NSP-positive SCLCs, NCAM and synaptophysin could be detected in virtually all tumour cells, while Leu-7 was present in three and chromogranin A and neurofilament protein immunoreactivity was found in one case.

**Squamous cell carcinoma**

The total group of 52 cases of squamous cell carcinoma tested consisted of a group of 22 cases preselected from a larger series (approximately 200 cases) on the basis of their positivity for NCAM and/or neurofilaments.15 The remaining 30 cases of squamous cell carcinoma were chosen on the basis of the absence of NCAM and neurofilaments.15 When examined for the complete spectrum, 28 of the 52 cases showed neuroendocrine markers (see Table III) ranging from one marker (10/28) to two (9/28), three (6/28) or four neuroendocrine markers (3/28) present. In these squamous cell carcinomas, NSP-A expression was observed in 16 out of 52 cases (see Table I), with only focal immunoreactivity in all these positive cases (Figs 2A and 2E). NSP-C could be detected in two of the 52 cases (Fig. 2B), one of which was also positive for NSP-A (cf. Figs 2A and 2B). With the exception of three tumours, NSP-positive cases showed the presence of other neuroendocrine markers. Most of these showed NCAM positivity (Figs 2C and 2H) next to NSP-A expression (10 of 16 NSP-A positive cases). In contrast, NCAM was found to be the only neuroendocrine marker detected in seven cases. Synaptophysin (Fig. 2F) and Leu-7 (Fig. 2G) expression was observed in seven cases, while chromogranin A was detected in two squamous cell carcinomas. Five cases of squamous cell carcinoma were shown to express at least one of the neurofilament subunits (Fig. 2D), which always co-occurred with at least one other neuroendocrine markers.

**Adenocarcinoma**

Six of the 18 cases of adenocarcinoma tested exhibited neuroendocrine features (see Table IV), ranging from
Fig. 2—Immunoreactivity patterns of two squamous cell carcinoma cases. The tumour shown in A–D is characterized by RNL-2 for NSP-A (A), RNL-4 for NSP-C (B), RNL-1 for NCAM (C), and MNF for neurofilament proteins (D). The tumour in E–H is characterized by MON 160–162 for NSP-A (E), polyclonal anti-synaptophysin (F), HNK-1 for Leu-7 (G), and RNL-1 for NCAM (H). The bar indicates 40 μm in A–C, 50 μm in D, and 55 μm in E–H.
one marker (4/6) to two (1/6) or three markers (1/6) present. NSP-A expression was observed in two of these cases, while strong NSP-C expression was seen in one case. Two cases showed the presence of synaptophysin, while three adenocarcinomas contained Leu-7. No neurofilament expression was found.


discussion

NSP-reticulon expression has been examined in the main subtypes of pulmonary neoplasia and was studied in relation to the expression patterns of other, conventional neuroendocrine markers. Comparison of the staining patterns of NSP-A with NSP-C, on the one hand, and of these NSP subtypes with the conventional neuroendocrine markers, on the other, has revealed three findings. Firstly, a high concordance exists between expression of NSP-A and NSP-C in neuroendocrine tumours of the lung, while in non-SCLC, NSP-A occurs at a much higher frequency than NSP-C. Secondly, in neuroendocrine tumours, NSP-A and NSP-C expression correlates well with NCAM and synaptophysin, while their independence of the presence of neurosecretory vesicles is reflected by the presence of both NSP-A and NSP-C in SCLCs, which are mainly negative for chromogranin A. Strikingly, NSP-reticulons were often absent in atypical carcinoids. Thirdly, in non-SCLC showing indications of a neuroendocrine immunophenotype, NSP-A expression is virtually always associated with one or more (up to three other) neuroendocrine markers.

Concordance between expression of NSP-A and NSP-C

Of the typical neuroendocrine tumours expressing NSP-A (13 cases), ten cases also expressed NSP-C, while, conversely, ten of the 11 NSP-C positive cases also contained NSP-A. This indicates a high concordance in expression between NSP-A and NSP-C in the neuroendocrine tumours. In the non-SCLCs, however, NSP-C is only co-expressed in a few cases of NSP-A positive tumours (two out of 18).

We have previously shown2–4 that NSP-A and NSP-C are to some extent differentially expressed in cell lines derived from neuroendocrine lung cancers, and we wondered whether such a disparity would also occur in their solid lung tumour counterparts. However, as mentioned above, in the typical neuroendocrine tumours only a few cases were observed with differential expression patterns of NSP-A and NSP-C.

Comparison of NSP-A and NSP-C with NCAM

The extent of expression of NSP-A and/or NSP-C approximates that of NCAM, which is positive in virtually all typical neuroendocrine cases. In this respect, it is worthwhile mentioning that co-expression of NSP-A and NCAM in SCLC cell lines, as well as non-SCLC-NE cell lines, has recently been demonstrated.4 However, in these cultures no complete overlap was seen between these markers at the single cell level, NSP-reticulons being in general less abundant than NCAM. A similar observation was made in the solid neuroendocrine tumours, in which often only a fraction of NCAM-positive cells appeared to contain NSP-reticulons. Although previous studies15 described the high sensitivity of NCAM as a marker for neuroendocrine differentiation, the specificity of NCAM for neuroendocrine cells has also been disputed. Kibbelaar et al.16 detected NCAM in cells lacking dense core granules and concluded that NCAM positivity is not absolutely specific for neuroendocrine differentiation. Furthermore, NCAM expression has been found in a range of non-neuroendocrine cells.14,17 We have found a considerable number of cases of non-SCLC with NCAM positivity not supported by the presence of another neuroendocrine marker, while virtually all NSP-A positive cases were found to contain one or more of the other neuroendocrine markers. Therefore, detection of NSP-A provides a valuable adjunct to NCAM in the precise diagnosis of neuroendocrine differentiation in lung cancer.

Comparison of NSP-A and NSP-C with markers of neurosecretory granules

Previous studies have shown that synaptophysin is a useful marker in neuroendocrine pulmonary tumours of all grades,18,19 although several studies have reported variable synaptophysin expression in SCLC.10,18,20 In cell cultures of SCLC, synaptophysin expression

Table IV—Immunophenotypes of adenocarcinomas of the lung

<table>
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<tr>
<th>AC cases</th>
<th>NSP-A</th>
<th>NSP-C</th>
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<th>Leu-7</th>
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</table>

AC=adenocarcinoma; NCAM=neural cell adhesion molecule; Syn=synaptophysin; CgA=chromogranin A; NF=neurofilament proteins. *F+, focal positivity in groups of cells or scattered cells; D+, virtually all cells diffusely positive; –, no staining.
appeared to be very useful for the detection of neuroendocrine differentiation, even in the variant-SCLC type, which in general lacks neurosecretory vesicles. On the basis of results in lung cancer-derived cell lines, NSP-reticulons were considered to be more sensitive markers for neuroendocrine differentiation than synaptophysin. However, in the underlying study, synaptophysin was found to be a slightly more sensitive marker than NSP-reticulons in typical neuroendocrine tumours. In general, it can be stated that NSP-positive neuroendocrine tumours are always positive for synaptophysin, while the opposite is true in a slightly smaller number of cases. In particular, atypical carcinoids were often found to be negative for NSP-reticulons, while synaptophysin was detected more often. The absence of NSPs as opposed to other neuroendocrine markers in atypical carcinoid may be of diagnostic relevance, as these tumours require extensive surgical therapy, whereas SCLC is treated with combined chemo- and radiotherapy and the typical carcinoid tumour is adequately treated with local excision. More cases of this tumour type have to be examined, however, to reach a convincing conclusion in this respect. Strikingly, in non-SCLC, the frequency of NSP-A expression is much higher than synaptophysin (18 vs. nine cases). In these malignancies, only four cases showed co-expression of these two markers, while synaptophysin occurred independently of NSP-reticulons in five of the nine positive cases. Also, non-SCLC-NE cell lines showed a broader expression of NSP-reticulons, compared with synaptophysin.

It is known that SCLCs express chromogranin A in only a low percentage of cases, merely due to their low granule content. Indeed, we found a decreasing fraction of cases positive for chromogranin A, with the predicted decrease in granule content going from carcinoid to SCLC (see Table II), while non-SCLC showed sporadic expression. It may be obvious that chromogranin A has to be considered a relatively insensitive, but specific marker for neuroendocrine differentiation in lung cancer.

Reliable detection of neuroendocrine differentiation in non-SCLC

Several studies have questioned whether immunohistochemical detection of neuroendocrine features in non-SCLC reflects a histologically mixed phenotype. It is beyond doubt that caution has to be taken in the interpretation of the usefulness of certain neuroendocrine markers, since several of these are not restricted to neuroendocrine tissues. For instance, neuron-specific enolase is not a reliable neuroendocrine marker, because it has been found in other than neural and neuroendocrine tissues. On the other hand, although highly specific, markers for neurosecretory granules often have a limited sensitivity, caused by the low neurosecretory granule content in poorly differentiated tumours. Such granules have been detected by electron microscopy in small numbers in non-neuroendocrine tumours. All these studies show that the individual criteria for identification of neuroendocrine differentiation are not absolute and should therefore be combined whenever possible. The underlying study shows that NSP-reticulons may play an important role in the detection of neuroendocrine differentiation in otherwise non-neuroendocrine malignancies. The fact that several NSP-antibodies are reactive with routinely processed tumour samples will allow retrospective studies into this clinically important aspect.

Research efforts are now concentrating on the importance of neuroendocrine differentiation in non-SCLC, since neuroendocrine differentiation is often presumed to be correlated with aggressive growth and response to chemotherapy. The studies dealing with this question have, however, reached conflicting conclusions. Several studies have indeed shown that non-SCLC-expressing neuroendocrine markers have a poorer prognosis than typical non-SCLC, while others have shown no correlation between neuroendocrine differentiation and survival. Moreover, some studies have shown that non-SCLCs with neuroendocrine differentiation have a better response to chemotherapy, although not always resulting in survival advantage. An extended study of NSP-A and NSP-C expression in correlation with clinical behaviour will be necessary to reveal whether these independent neuroendocrine markers are better prognosticators than the conventional neuroendocrine markers.

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