

Onconeural antibodies in sera from patients with various types of tumours

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Abstract

Purpose We assessed the frequency and levels of onconeural antibodies in 974 patients with various types of tumours, but without apparent paraneoplastic neurological syndromes (PNS).

Patients and methods We included patients with the following tumours: 200 small-cell lung cancer (SCLC) patients, 253 breast cancer patients, 182 ovarian cancer patients, 266 uterine cancer patients and 73 thymoma patients, as well as 52 patients with PNS and cancer and 300 healthy blood donors. Sera were screened for amphiphysin, CRMP5, Hu, Ma2, Ri and Yo antibodies using a multi-well immunoprecipitation technique.

Results The most frequently detected antibodies were Hu followed by CRMP5. Ma2, Yo, amphiphysin and Ri antibodies were less common, but each was found at similar frequencies. Onconeural antibodies were present at similar levels in sera from the PNS control group and from cancer patients. Hu antibodies were rare in cancers other than SCLC. CRMP5 was the only antibody found in patients with thymoma and this antibody was more common among patients with thymoma than in other tumour patients. With one exception, coexisting antibodies were only found in

patients with SCLC. The presence of onconeural antibodies in SCLC patients was not associated with prolonged survival.

Conclusion Onconeural antibodies are associated with various types of tumours suggesting that all antibodies should be included in the serological screening for possible PNS. The levels of onconeural antibody are not sufficiently sensitive to discriminate between cancer patients with PNS and those without.

Keywords Onconeural antibodies · Cancer · Paraneoplastic neurological syndrome · Immunoprecipitation

Introduction

Anti-tumour immune responses to neuronal antigens expressed by cancer cells may result in marker antibodies that can be detected in serum. Although these “onconeural antibodies” are often associated with paraneoplastic neurological syndromes (PNS), they can also be found in cancer patients without neurological symptoms [1–7].

The six best characterised and most prevalent onconeural antibodies are anti-amphiphysin, anti-CRMP5, anti-Hu, anti-Ma2, anti-Ri and anti-Yo (for a review see Graus et al. [8]). The ranking order of these antibodies in routine screening of patients with possible PNS is anti-Hu ~ anti-CRMP5 > anti-Yo > anti-amphiphysin > anti-Ri, while the frequency of Ma2 is not known [3]. Coexisting antibodies are often present [3].

Onconeural antibodies are most commonly detected using immuno-histochemistry and immune blots with neuronal extracts or recombinant proteins. We established a sensitive immunoprecipitation technique for detecting

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onconeural antibodies [4–7]. This study aimed to apply this immunoprecipitation technique to examine the prevalence of amphiphysin, CRMP5, Hu, Ma2, Ri and Yo antibodies in sera from patients with small-cell lung cancer (SCLC), thymoma, breast-, ovarian- or uterine cancer.

Patients and methods

Patients and controls

Sera from 200 patients with SCLC, 253 patients with breast cancer, 182 patients with ovarian cancer, 266 patients with uterine cancer and 73 patients with thymoma were included. Due to a shortage of some of the breast cancer sera, one patient was not analysed for Hu and CRMP5 antibodies and four were not analysed for Ma2 antibodies. Similarly, six thymoma patients were not analysed for Hu and Ma2, seven were not analysed for Yo and amphiphysin and eight were not analysed for Ri antibodies because of shortage of sera. Sera were obtained before start of treatment, except for 62 metastatic breast cancers, and kept frozen until use.

The SCLC patients have previously been tested for anti-Hu and anti-VGCC [4], anti-Ri [6], anti-CRMP5 [7], and survival data were available. Patients with breast cancer, and some of the patients with ovarian cancer, have previously been tested for anti-Ri and anti-Yo [5, 6]. In the antibody-positive SCLC patients, medical records were reviewed for possible PNS by an oncologist. In the antibody-positive breast, ovarian or uterine cancer patients, records were reviewed by a neurologist. All thymoma patients had myasthenia gravis, and these patients have previously been tested for CRMP5 [7].

Those sera that were tested previously for any of the antibodies were retested in this study. The patients gave informed consent for inclusion in the study, which was approved by the local medical research ethics committee.

Samples from 300 blood donors at the Haukeland University Hospital were used as normal controls. No clinical data were available for the blood donors. We also included 52 onconeural antibody positive sera from patients with known cancer and classical PNS [8].

In vitro transcription–translation (ITT) and immunoprecipitation

The cDNA for the onconeural proteins were placed into expression vectors, with a T3 or T7 promoter. The vectors containing Hu, Yo, Ri and CRMP5 have been described earlier [4–7]. The amphiphysin cDNA and the Ma2 cDNA containing plasmids were generous gifts from Peter Sillevius Smitt (Erasmus University Medical Center, Rotterdam,

The Netherlands) and Raymond Voltz (Klinikum Grosshadern, Munchen, Germany), respectively. ITT was performed as previously described [7].

Patient sera were screened by immunoprecipitation, employing [³⁵S]-labelled onconeural antigens (20,000–30,000 cpm/well) and a 1:20 dilution of sera [5].

Pooled sera from 100 blood donors were used as a negative control. A polyclonal rabbit antibody (Eurogentec s.a., Seraing, Belgium) against two synthetic peptides of the actual human onconeural protein sequence was used as positive control in each assay, except for the Ri assay where a positive serum from a patient with cancer and PNS was used. The peptides were coupled to KLH (keyhole limpet haemocyanin). Control tests using preimmune rabbit sera were negative.

Each patient serum sample was run in triplicate and the mean value of these used. The results were expressed as an index: $(\text{cpm sample} - \text{cpm negative control}) / (\text{cpm positive control} - \text{cpm negative control}) \times 1,000$. An upper limit based on the mean index of 300 blood donors + 3 SD was calculated for each of the six antibodies. Patient samples with an index greater than this cut-off value were considered as positive. Positive results were verified by repetitions of the immunoprecipitation experiment and each repetition had to yield an index above cut-off to include the sample as a positive.

None of the antibody-positive sera reacted when immunoprecipitation was run with a template negative ITT-product. This excludes alternative explanation for immunoprecipitation of the positive samples and confirms the specificity of the technique.

Statistical analysis

The Kaplan–Meier survival curves were used to estimate survival after SCLC diagnosis for the onconeural antibody-positive and antibody-negative patient groups. For univariate comparing of survival curves the Log-Rank test was used. The Cox Proportional Hazard Model was used to study the effect of several factors simultaneously on survival and to estimate hazard ratios. The analyses were performed with SPSS version 14 (SPSS Inc, Chicago, IL).

Results

Controls

The following cut-off index based on the 300 blood donors was used: Hu: 262, CRMP5: 213, amphiphysin: 145, Yo: 174, Ri: 129 and Ma2: 263. Four of the 300 controls had positive antibody indices; the remaining 296 controls were below the cut-off limit. The distributions of blood donor indices for each of the six antibodies are shown in Fig. 1.

Fig. 1 Histograms showing the distribution of antibody indices for the 300 blood donors including the four positives

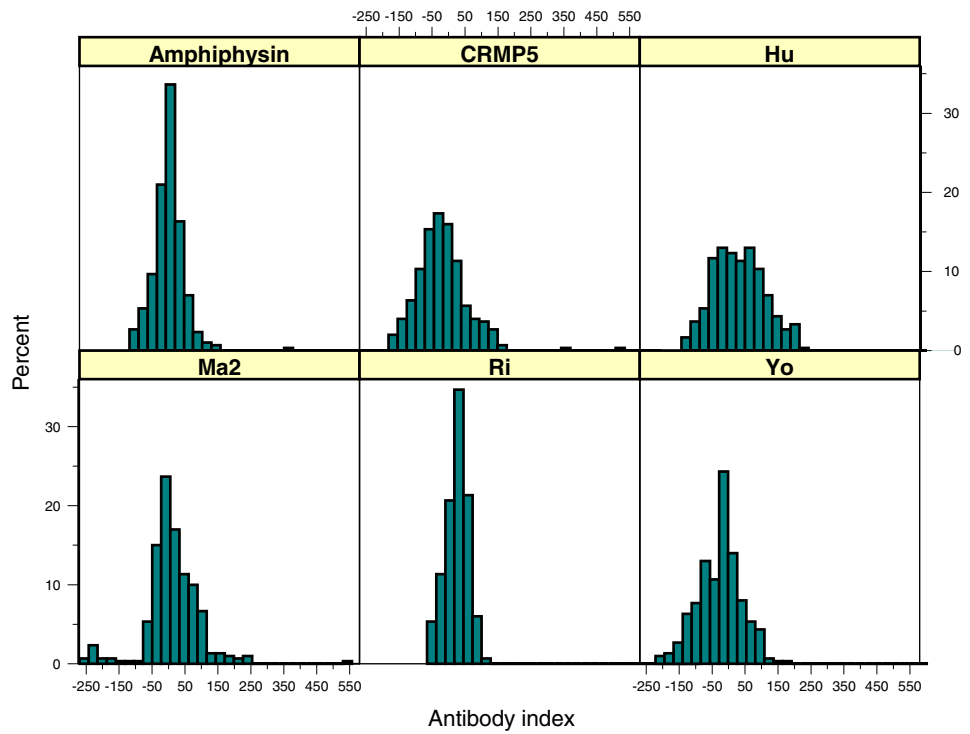


Table 1 Prevalence of onconeural antibodies in patients with various tumours, blood donors and PNS controls

	Hu	CRMP5	Amph.	Yo	Ri	Ma2	Co. ex.	Total pos./ca.
SCLC (<i>n</i> = 200)	45 (22.5%)	10 (5%)	5 (2.5%)	1 (0.5%)	3 (1.5%)	2 (1%)	9 (4.5%)	57 (28.5%)
Breast ca. (<i>n</i> = 253)	1 (0.4%)	3 (1.2%)	2 (0.8%)	3 (1.2%)	1 (0.4%)	4 (1.6%)	0	14 (5.5%)
Ovarian ca. (<i>n</i> = 182)	2 (1.1%)	3 (1.6%)	0	3 (1.6%)	1 (0.5%)	0	1 (0.5%)	8 (4.4%)
Uterine ca. (<i>n</i> = 266)	1 (0.4%)	3 (1.1%)	0	1 (0.4%)	1 (0.4%)	2 (0.8%)	0	8 (3.0%)
Thymoma (<i>n</i> = 73)	0	9 (12.3%)	0	0	0	0	0	9 (12.3%)
Ab. freq. (<i>n</i> = 974)	49 (5.0%)	28 (2.9%)	7 (0.7%)	8 (0.8%)	6 (0.6%)	8 (0.8%)	10 (1.0%)	96 (9.9%)/106 (10.9%)
Blood donors (<i>n</i> = 300)	0	2 (0.7%)	1 (0.3%)	0	0	1 (0.3%)	0	4 (1.3%)
PNS controls (<i>n</i> = 52)	22 (42.3%)	7 (13.5%)	4 (7.7%)	16 (30.8%)	2 (3.8%)	5 (9.6%)	4 (7.7%)	52 (100%)

Co. ex. coexisting antibodies in a sample (two or more antibodies), *total pos./ca.* the number of antibody-positive patients in each cancer group, *ab. freq.* the overall frequency of each antibody in these tumours. The total number of antibody-positive patients (96) is different from the total number of antibody positives (106) because of coexisting antibodies in ten patients

Four of the sera in the PNS control group had two antibodies (3 with Hu and CRMP5 and 1 with Hu and amphiphysin). The following cancers were present in this group: 25 lung (mostly SCLC), 14 ovarian, 9 breast, 3 prostate and 1 testis.

Cancer patients

The prevalence of onconeural antibodies in cancer and controls are presented in Table 1, while the levels of these antibodies are shown in Fig. 2. Patient samples with similar indices of an antibody might be difficult to distinguish in the plots. Nine of the 45 Hu positive SCLC patients had one or more antibodies in addition to

anti-Hu: 5 CRMP5, 1 amphiphysin, 1 Ri, 1 Ma2 and 1 both CRMP5 and amphiphysin. One patient with ovarian cancer had coexisting antibodies (Hu and CRMP5). Clinical information was not available for one antibody-positive breast cancer patient, but none of the other antibody-positive cancer patients had apparent PNS with the exception of the thymoma patients who all had MG.

The presence of one or more of the onconeural antibodies in the SCLC patients did not correlate with survival (*P* = 0.27, univariate model). A multivariate Cox-regression model, adjusting for age, extent of disease and lactate dehydrogenase, gave the same result (*P* = 0.24). Figure 3 illustrates Kaplan–Meier survival curves for those positive or negative for such antibodies.

Fig. 2 Plots of indices for antibody-positive sera from patients with SCLC, breast cancer (BC), ovarian cancer (OC), uterine cancer (UC) and thymoma (with MG) (T), as well as from blood donors (BD) and PNS controls. The cut-off value is indicated as a line in the figure

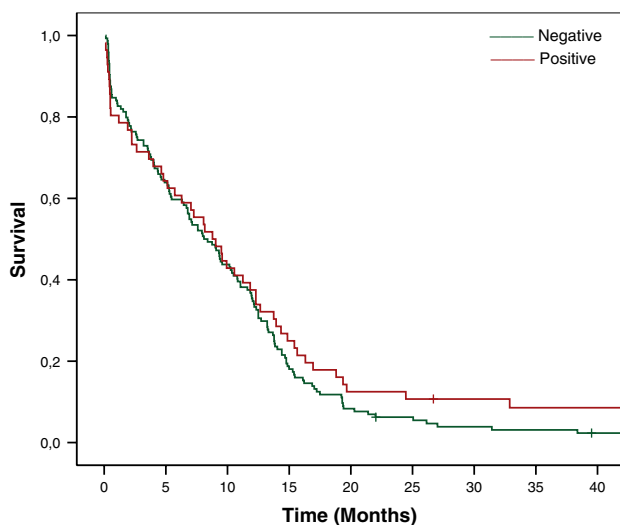
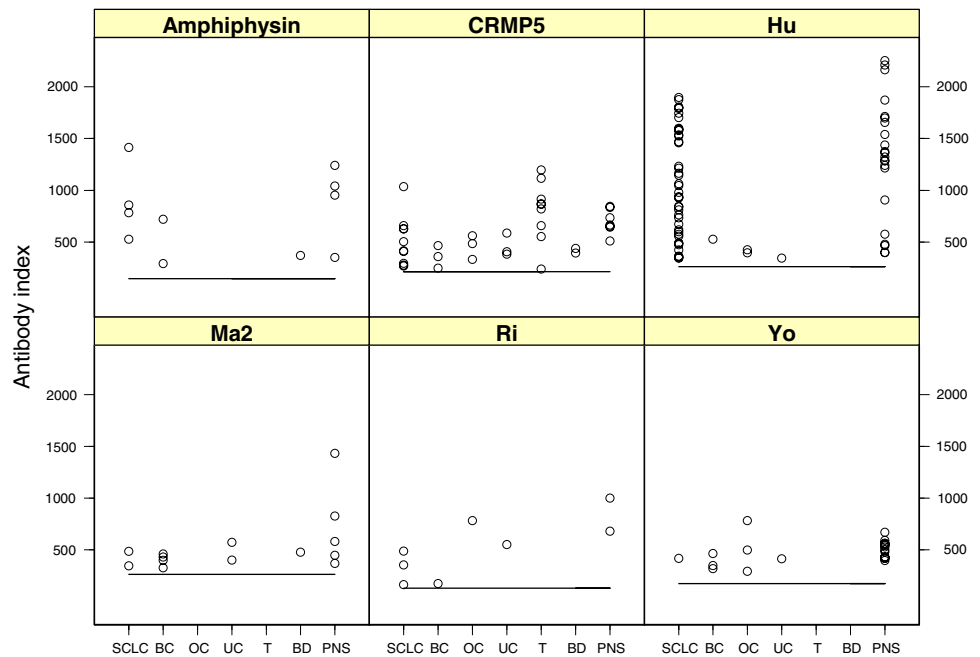


Fig. 3 Kaplan–Meier survival curves for antibody-positive and antibody-negative SCLC patients

Discussion

This study is, to our knowledge, the largest tumour material to be systematically analysed for all of the most common onconeural antibodies. Further, we used an immunoprecipitation technique that is more sensitive in detecting such antibodies than the commonly used methods for this purpose [4–7]. This technique is based on [³⁵S]-labelled recombinant protein made in a mammalian cell lysate, and the protein, which is likely to have native conformation, allows the antibodies to detect both linear and conforma-

tional epitopes. In addition, it is a fluid-phase technique in which the protein is free in solution, leaving every part of the antigen accessible to the antibody. This technique combines high specificity and sensitivity with a high capacity for analysis. Each antibody assay has individual peptide sera as positive control (with the exception of the Ri assay, which has a patient serum as positive control), and the index value is therefore not comparable from one antibody to another.

In related immunoprecipitation studies, mean + 2 SD [9], 3 SD [10, 11] and 4 SD [12] have been employed to calculate cut-off values for an antibody, based on the results of 91, 85 and 70 blood donors, respectively. In the present study, we have used a cut-off value based on the mean index of 300 blood donors +3 SD, for all six antibodies. We are not aware of any other similar studies that included such a high number of blood donor controls. Due to the large number of controls, we were able to set the cut-offs higher than in previous studies [4–6] and this explains why some formerly low-positive samples now fall below the cut-offs and are therefore considered negative.

The 300 blood donors show a close-to-normal distribution for each antibody analysed, except for four outliers. The indices of these were clearly elevated compared to the other blood donors and were in the same range as the indices of the low-positive cancer patients and the low-positive PNS controls. Two of these blood donors had CRMP5 antibodies, one had amphiphysin and one had Ma2 antibodies. Since no clinical information was available for the blood donors, it is not known whether any of them have had occult tumour. Onconeural antibodies have, however, previously been described in the absence of a tumour [13, 14].

In total, 28.5% of the SCLC, 5.5% of the breast cancer, 4.4% of the ovarian cancer 3.0% of the uterine cancer and 12.3% of the thymoma patients had at least one onconeural antibody. The total frequencies of each antibody in sera from these patients were 5.0% anti-Hu, 2.9% anti-CRMP5, 0.8% anti-Ma2, 0.8% anti-Yo, 0.7% anti-amphiphysin and 0.6% anti-Ri, respectively. No antibody-positive SCLC, breast (information is lacking for one patient), ovarian or uterine cancer patients had an apparent PNS. However, we cannot exclude that some of the patients had minor signs of PNS, since none of these patients were examined by a neurologist. All our thymoma patients had myasthenia gravis. In a previous study, CRMP5 antibodies were less prevalent in thymoma patients without myasthenia gravis compared to thymoma patients with myasthenia gravis (7 vs 17%) [15]. The frequency of CRMP5 antibodies in our thymoma patients may therefore be relatively higher than in the other cancer materials used, in which none had a known PNS.

Hu antibodies are most commonly associated with SCLC and paraneoplastic encephalomyelitis [16]. SCLC patients with no PNS also have Hu antibodies [1, 4], but the prevalence of Hu antibodies in other cancers known to be associated with onconeural antibodies have not been evaluated earlier. We found Hu antibodies rarely in patients with breast, ovarian, and uterine cancer, but not in thymoma, indicating that Hu antibodies are mostly restricted to SCLC.

In patients presenting with neurological symptoms and CRMP5 antibodies, SCLC and thymoma are the most frequently encountered tumours [17]. The prevalence of CRMP5 antibodies in thymoma with or without myasthenia gravis has been reported [15]. However, to our knowledge, no study has investigated the prevalence of CRMP5 antibodies in other tumours, except one study describing CRMP5 antibodies in 9% of SCLC patients using a lysate of CRMP5 fusion protein [18]. CRMP5 antibodies were more frequent in patients with thymoma and myasthenia gravis than in patients with SCLC, whereas CRMP5 antibodies were less frequent in breast, ovarian and uterine cancers (1–2%).

Patients with various types of PNS and tumours might develop amphiphysin antibodies [19], but the most common cancer types associated with these antibodies are SCLC and breast cancer [20]. The presence of amphiphysin antibodies has not been investigated in larger cancer materials without PNS, except for one study in which 1.4% of SCLC patients without PNS had amphiphysin antibodies [21]. In our study, amphiphysin antibodies were more common in patients with SCLC than in patients with breast cancer, whereas patients with ovarian or uterine cancer did not have amphiphysin antibodies.

Yo antibodies are most common in patients with paraneoplastic cerebellar degeneration (PCD) and breast or

ovarian cancer [22]. Yo antibodies have also been described in 2.2% of ovarian cancer patients with no PNS [2], which is a slightly higher prevalence than 1.6% found in this study. Except for one case with SCLC and one case with uterine cancer, we found Yo antibodies only in patients with breast or ovarian cancer.

Ri antibodies have been found in patients with different neurological disorders and usually lung- (SCLC or non-SCLC) or breast cancer [23]. Ri antibodies have also been found in 4% of patients with ovarian cancer but no PNS [2]. We found Ri antibodies in only 0.5% of the ovarian cancer patients, 1.5% of the SCLC patients and 0.4% of the breast and uterine cancer patients, respectively.

The higher frequency of antibody positivity in these studies may be explained by the use of apparently unpurified recombinant protein or extract of brain tissue to the search for CRMP5 [18], Yo and Ri [2] antibodies. The unpurified protein does not have adequate specificity and may thus be associated with false-positive results.

Ma2 is usually associated with paraneoplastic limbic or brainstem encephalitis and testicular cancer [24]. To our knowledge, there has been no report on the frequency of Ma2 antibodies in cancer patients without PNS. In our material, Ma2 antibodies were found with approximately the same frequency as Yo antibodies in breast cancer patients (1.6 vs 1.2%), and Ma2 antibodies were also present in a few patients with SCLC or uterine cancer.

We found that coexisting antibodies are common in patients with SCLC, but not in the other tumour patients investigated. Nine (4.5%) of the SCLC patients had two or more types of antibodies. CRMP5 antibodies coexisted more often with Hu antibodies than alone, in accordance with the findings of Pittock et al. [3].

Both low and high levels of Yo and Ri antibodies have been found in patients with ovarian cancer without PNS [2], while mostly low levels of Hu antibodies were found in SCLC patients having no PNS [1]. In our study, onconeural antibodies were present at similar levels in sera from patients with and without PNS. The level of onconeural antibodies is not therefore an indication of the presence of a PNS.

We have previously found that the survival of patients with SCLC is not associated with the presence of Hu [4], Ri [6] or CRMP5 [7] antibodies. The results of this study similarly indicate that the presence of one or more of the six onconeural antibodies does not predict survival of patients with SCLC.

In summary, the results show that patients with a given onconeural antibody may have different types of tumours, and patients with certain tumours may have different onconeural antibodies. This indicates a need for analysing all onconeural antibodies in routine screening of patients with possible PNS. Further, onconeural antibodies are

commonly associated with tumours without PNS, regardless of antibody levels, emphasising that even high serum levels of these antibodies are not necessarily associated with PNS.

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