Long-Term Analysis of Ab-2 (Clone SN3b) Immunoreactivity as a Prognostic Factor in Breast Carcinoma

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Summary

Background: CD24 expression has been described as a significant prognostic factor in multiple solid tumours. Most of these studies have, however, been undertaken using the Ab-2 antibody (clone SN3b), which detects a CD24-associated carbohydrate, and not the CD24 protein itself. Although its biological identity remains unclear, its prognostic significance means that detection of this carbohydrate may, nonetheless, be clinically relevant. **Methods:** 133 breast carcinomas were selected (pT1–2 pN0–2 M0, no secondary carcinoma, no contralateral carcinoma) from a previous SN3b expression study on a larger cohort of breast carcinomas. After updating data on follow-up observations, we carried out univariate and multivariate analysis of the prognostic significance of SN3b for total and breast cancer-specific survival. **Results:** A statistically significant correlation between cytoplasmic SN3b immunoreactivity and positive node status was found. Cytoplasmic SN3b also has node status-independent prognostic significance. Total survival exhibits a statistically significant dependency on cytoplasmic SN3b even for pN0 cases. **Conclusion:** The independent prognostic value of CD24 as detected by Ab-2/clone SN3b could replace the diagnostic axillary dissection in breast carcinoma patients if this was confirmed in further studies. Also, clarifying the exact epitope of this interesting antibody is more than warranted.

Introduction

In 2003, our group established that overexpression of CD24 in invasive breast carcinoma is prognostically unfavourable [1]. Our initial study used the monoclonal CD24 antibody Ab-2 (clone SN3b, Neomarkers (Fremont, CA, USA)), which was technically very robust and subsequently employed by innumerable groups to detect CD24, even though its precise epitope was – and still is – elusive. In a later study [2], we compared the CD24 immunoreactivity patterns of clone SN3b with those of clone SWA11, which have been well characterised as specific for the CD24 protein core. This comparison revealed only a partial overlap between both antibodies. It is probable that clone SN3b detects sialic acid on O-linked glycans, which can also occur independently of the CD24 protein backbone. This implies that mAb SN3b is not strictly a CD24-specific antibody, which explains discrepancies with other studies, although it presumably accounts for the major component of 'CD24' immunoreactivity. This is even more of interest since SWA11 failed to show a statistically significant prognostic value, although a weak positive trend was discernible.

Our original study published in 2003 had several minor shortcomings. First, it encompassed patients from different clinics who had been diagnosed at the pathology institute of the Charité; secondly, it was based on patient data covering an average follow-up observation period of only 65 months (5—129 months); and thirdly, it lacked access to additional information on secondary tumours, etc. The acquisition of markedly prolonged follow-up data and additional patient information prompted us to reanalyse the original CD24/SN3b expression data of the patients from a single centre (Breast Cancer Centre Charité).

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Patients and Methods

Patients

From our previous study, we identified 139 cases with primary breast carcinoma. These had undergone surgery in the surgical unit on the central Charité campus and were diagnosed at the Institute of Pathology of the Charité. 2 of these cases had other previous or concurrent malignancies and were therefore excluded from the analysis. 5 patients who subsequently developed other malignant tumours remained in the analysis. A further 4 patients who had previously been diagnosed with a contralateral breast carcinoma prior to the breast carcinoma under investigation were also excluded from the study. 4 patients who developed a simultaneous (i.e. within 6 months) contralateral breast carcinoma and 7 patients who subsequently developed a contralateral breast carcinoma were not excluded from the study. In total, 133 cases remained in the analysis. Of these, 127 cases were M0 and 6 cases were M1.

Follow-Up Data

Total survival up to a cut-off date of 30.06.2007 was analysed. The death rates of a previously published analysis of all patients between 1984 and 1998 were underestimated since they were exclusively based on follow-up data from a single clinical tumour register that contained data established solely on further consultations with patients. These data were, therefore, supplemented with data on survival times from the Joint Cancer Register of the States of Berlin, Brandenburg, Mecklenburg-West Pomerania, Saxony-Anhalt and the Free States of Saxony and Thuringia (GKR) and from the Berlin Resident Registration Office [3].

Since no data on continued survival were available from the resident registration offices in the other German states, in particular those in the former East Germany, the number of deaths of patients from these states was weighted more heavily despite the identical recording period. This is because data from other resident registration offices, if they had been available, would have supplemented data from the clinical register prior to the cut-off date for the analysis for those patients who were no longer attending hospital consultations.

Immunohistochemistry

CD24 immunostaining was conducted as described previously [1]. Briefly, freshly cut paraffin sections were dewaxed with xylene and gradually hydrated. Antigen retrieval was achieved by pressure cooking in 0.01 M citrate buffer for 5 min. The primary CD24 antibody (clone SN3b; Neomarkers, Fremont, USA) was diluted 1:100 and incubated at room temperature for 2 h. The conventional labelled streptavidin-biotin method was used for detection with alkaline phosphatase as the reporting enzyme and chromogenic development with Fast-Red. Finally, the slides were briefly counterstained and mounted.

In the initial study we kept our scoring system of the CD24 staining as simple as possible to enhance the reproducibility of our findings in future studies [1]. We evaluated the membranous and the cytoplasmic staining intensity of CD24 separately and scored the staining intensity as negative (0), or weakly (1+), moderately (2+) or strongly (3+) positive.

Table 1. Association of CD24 (clone SN3b) with clinicopathological parameters

Characteristic	CD24/SN3b cytoplasmic				Approx. significance[a]
	0	1	2	3	
pT					
pT1	22	30	24	10	
pT2	6	17	12	7	0.256
pT3	1	0	0	1	
pT4	1	0	2	0	
pN					
pN0	21	21	15	7	
pN1	8	21	20	8	0.013
pN2	1	5	3	3	
Histological grade					
G1	13	14	10	5	
G2	14	21	20	10	0.366
G3	3	10	5	2	
Oestrogen receptor					
Negative	5	18	11	8	0.126
Positive	24	28	27	9	

[a]Ordinal by ordinal gamma

Table 2. Univariate Cox regression for single prognostic parameters (overall survival)

Prognostic parameter	Statistical significance	For the analysis:			Exp(B)
		Cases with missing data	Censored cases before the earliest event in a stratum	Available deaths	
pT	0.007	0	3	40	1.569
pN	0.002	0	3	39	2.208
G	0.014	6	3	37	1.765
ER	0.082	3	3	38	0.566
CD24/SN3b	0.003	0	3	40	1.661

ER = oestrogen receptor, Exp(B) = hazard ratio.

Results

The mean observation period was 96.1 months (minimum 1 month, maximum 192 months). Follow-up data received from the clinical cancer registry were supplemented with data from Berlin Resident Registration Office records and the cancer registry of the new federal states of Germany (GKR). In total, 40 deaths were recorded. Because the first event (death) occurred after 6 months, 2 cases for which the follow-up period was only 1 month and 1 case for which it was 5 months were discarded, leaving 130 patients in our analysis. Due to missing data the number of cases analysed for some individual parameters was lower.
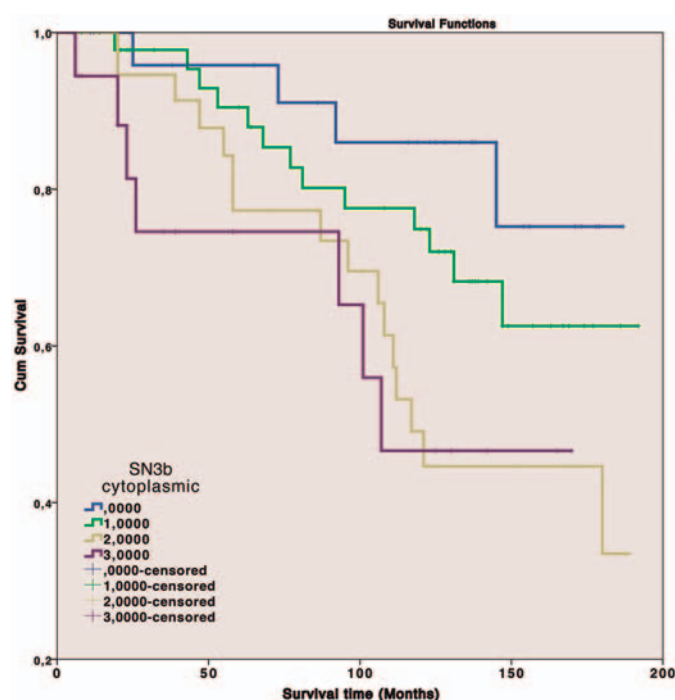
Fig. 1. Survival times from the Joint Cancer Register of the federal states of Berlin, Brandenburg, Mecklenburg-West Pomerania, Saxony-Anhalt, the free states of Saxony and Thuringia (GKR) and from the Berlin resident registration office.

Table 3. Multivariate Cox regression of CD24 expression and clinicopathological parameters – overall survival

		Sig.	Exp(B)
Step 1	pT	0.171	1.340
	pN	0.134	1.642
	G	0.061	1.625
	ER	0.895	0.952
	CD24/SN3bc	0.007	1.688
Step 2	pT	0.172	1.334
	pN	0.120	1.656
	G	0.041	1.644
	CD24/SN3bc	0.007	1.691
Step 3	pN	0.035	1.866
	G	0.051	1.621
	CD24/SN3bc	0.007	1.693

Table 4. Multivariate Cox regression of CD24/SN3b expression and clinicopathological parameters (pN0) – overall surviva

		Sig.	Exp(B)
Step 1	pT	0.170	4.728
	G	0.827	0.888
	ER	0.108	0.338
	SN3b-cytoplasmic	0.031	1.899
Step 2	pt	0.171	4.678
	ER	0.091	0.361
	SN3b-cytoplasmic	0.032	1.894
Step 3	ER	0.067	0.338
	SN3b-cytoplasmic	0.045	1.779

We found a significant association of CD24 expression with a positive nodal status (table 1). This association between pN and cytoplasmic SN3b was further tested using the gamma statistics, which is suitable for analysing associations between ordinal-scaled variables. The corresponding cross-table (table 1) showed a positive association between cytoplasmic SN3b and pN. A gamma test for no association between cytoplasmic SN3b and pN (gamma test) was rejected with a significance level of $p = 0.013$.

Survival Analyses

We conducted a univariate Cox regression to determine the prognostic value for overall survival of CD24 and the standard prognostic parameters (pT, pN, G and oestrogen receptor) (table 2). The prognostic significance of CD24/SN3b was further confirmed in a Kaplan Meier analysis (fig. 1; $p = 0.023$ (log rank test)).

To approach a multivariate setting, backward stepwise (Wald) Cox regression analysis using the above prognostic parameters was employed: of the 130 cases, 15 were omitted due to missing data, leaving 115 cases and 34 events. In the resulting model, cytoplasmic CD24/SN3b immunoreactivity was the strongest independent prognostic parameter (table 3).

Despite the small number of cases, we considered it of interest to examine total survival time as a function of cytoplasmic SN3b for pN0 cases only (table 4). As with the above calculations, 60 cases (64 minus 2 cases censored before the earliest event in the stratum and 2 cases with missing values) with 12 deaths were included in the calculation, which yielded a significance of $p = 0.045$ (backwards stepwise Cox regression). The same calculation for all pT1pN0 cases shows that the number of cases remains too small (data not shown).

Discussion

Our reanalysis revealed that cytoplasmic CD24/SN3b immunoreactivity is found to improve the prognostic accuracy further in terms of breast cancer-specific survival, even compared to nodal status. We are well aware that calculating breast cancer-specific survival is controversial due to diagnostic uncertainty in death statistics – as a result, for example, of death certificates not always being filled out correctly [4–6] or of treatment-induced premature deaths not directly caused by breast cancer. For this reason we deliberately used overall patient survival times. The results for the pN0 cases appear to support the validity of this method.

The increase in events detected using our data collection methodology of incorporating data from additional cancer registries is beneficial for this comparative analysis. Although the appropriateness of the Kaplan-Meier method can be questioned in cohorts with complex follow-up compositions like ours [3], we have still utilized this widely applied technique for approximation and the subsequent Cox analyses confirmed the prognostic value of CD24/SN3b. These results for cytoplasmic CD24/SN3b are even more interesting in light of recent data that led to discussions on the value of axillary lymph node dissection in cases in which several sentinel

lymph nodes (up to 2) are positive [7–9]. This could imply that the quantity of lymph node involvement would no longer be available as a prognostic factor.

Unfortunately, the number of cases in this study is too low to identify putative pT1pN0 cases by an evaluation of cytoplasmic CD24/SN3b. If this were possible, it would identify a subgroup of small tumours with no lymph node involvement that still have a poor prognosis. In addition, currently the idea that local lymph node dissection is not significant for further survival is under considerable discussion [10].

Clearly, a much larger study of well-characterized breast cancer cases is not only warranted but also urgently needed to elucidate the promising prognostic value of SN3b immunoreactivity, ideally in direct comparison to an antibody that recognizes the CD24 protein core (e.g. SWA11). CD24 began to attract the attention of cancer researchers a decade ago. It is overexpressed in the majority of human carcinomas and is often associated with a poorer prognosis [11]. Many of these studies were carried out with the SN3b antibody; therefore, it is time to investigate and annotate the SN3b epitope to understand its tumour-biological functions.

In conclusion, CD24/Ab-2 (clone SN3b) immunoreactivity is a strong prognostic factor in node-negative primary breast carcinomas and provides useful information supplementing that obtained from lymph node involvement.

Disclosure Statement

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