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Study of the Expression and Function of the Tumour-associated Antigen CaMBr1 in Small Cell Lung Carcinomas

Stefania Martignone, Sylvie Ménard, Amedeo Bedini, Adriano Paccagnella, Silvano Fasolato, Rosalia Veggian and Maria Ines Colnaghi

The expression of the epithelial antigen recognised by the MBr1 monoclonal antibody (CaMBr1) was studied on 161 small cell lung carcinoma (SCLC) biopsies. A correlation between the marker expression and the overall survival of the patients was found. To investigate the possible role of CaMBr1 in tumour aggressiveness, the *in vivo* and *in vitro* growth capabilities of different SCLC cell lines, in relation to the antigen expression, were analysed. The CaMBr1-positive cell lines displayed a higher growth potential in comparison to CaMBr1-negative cells. The biochemical nature of CaMBr1 was analysed in terms of enzyme sensitivity, molecular weight and comparison with other glycoproteins expressed by SCLC cells. The results indicated the trypsin sensitivity of the molecule, and sialic acid hiding of the CaMBr1 epitope. The increase of MBr1 reactivity after neuraminidase treatment suggests that the CaMBr1 epitope expressed in the SCLC cell line is carried by a sialoglycoprotein.
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INTRODUCTION

SMALL CELL lung cancer (SCLC), which represents 25% of lung malignancies, is characterised by high aggressiveness and early widespread metastases resulting in a very poor prognosis, despite initial sensitivity to chemotherapy [1].

Since limited disease at presentation guarantees long-term survival in only a few cases, several parameters have been investigated in order to obtain a better understanding of the tumour biology and to identify new prognostic indicators [2].

Important biological information has been obtained from SCLC-derived cell lines concerning the biochemical, immunological and cytogenetic phenotypes and oncogene amplification. Furthermore, analysis of the expression of molecules with particular biological significance using immunological techniques and employing relevant monoclonal antibodies (MAb), has been demonstrated to be useful in obtaining prognostic information [3, 4].

A preliminary study on a small series of SCLC biopsies indicated that the expression of a molecule recognised by the MAb MBr1 (CaMBr1) was significantly associated with a poor prognosis [5]. In breast cancer, the epitope identified by MBr1 is present on glycoproteins with heterogeneous molecular weight [6], as well as on a neutral glycolipid of the globo series (GL6) [7], whereas in SCLC it is associated with glycoproteins only [5].

In the present study, the prognostic significance of the MBr1 reactivity was further investigated on a larger series of SCLC biopsies. In addition, the biological function on cell growth and the biochemical nature of the molecule expressed on SCLC were analysed.

Correspondence to M.I. Colnaghi.
S. Martignone, S. Ménard, A. Bedini and M.I. Colnaghi are at the Experimental Oncology E, Istituto Nazionale Tumori, Via Venezian 1, 20133 Milan, Italy; and A. Paccagnella, S. Fasolato and R. Veggian are at the Medical Oncology Department, Padoa, Italy.
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MATERIALS AND METHODS

Monoclonal antibody

The origin and characteristics of the MBr1 MAb have been reported previously [6–8]. The MAb was used as ascitic fluid or purified from it by affinity chromatography on a protein A-Sepharose CL4B (Pharmacia, Uppsala, Sweden) column [9]. Leu7 and Leu19 were purified MAb from Becton Dickinson (Mountain View, California, U.S.A.).

Cell lines

Human SCLC cell lines NCI-H69, NCI-H128 and N592 were all provided by the American Type Culture Collection (ATCC). The human SCLC cell line POVD was kindly provided by Dr O. Pratesi (Istituto Nazionale Tumori, Milan).

The cell lines were all maintained in RPMI 1640 medium (Microbiological Associates, Walkersville, Maryland, U.S.A.) supplemented with 10% fetal calf serum (FCS), penicillin (100 μg/ml) and streptomycin (100 μ/ml).

Patients

The series included 161 tumours from patients operated upon and followed at the National Cancer Institute of Milan (INT), from patients belonging to an international study group on SCLC coordinated by Prof. K. Karrer (University of Vienna) and from patients of the Medical Oncology Department of Padoa. The numbers of MBr1-positive and MBr1-negative tumours were 68 and 93, respectively.

All patients had histologically confirmed SCLC and a known stage, treatment and survival. The majority of the biopsies were obtained from primary tumours [10] and additional cases from local or distant metastases.

Prior to surgery, the patients were treated with conventional chemotherapy consisting of cyclophosphamide, doxorubicin, etoposide, vincristine, cisplatin, iphosphamide. Radiation therapy was given in cases of non-radical resection and further chemotherapy was also given after surgical treatment.

The following parameters: type of treatment, age, sex and

stage of the disease, were homogeneously distributed in the two groups divided according to MBr1 activity.

Statistical methods

Association between antigen expression and stage of the disease was investigated by means of Mantel–Haenszel χ^2 test. Survival curves were drawn according to the Kaplan–Meier method [11]. As determined conventionally, $P < 0.05$ was considered to be significant. Zero time was considered the time of diagnosis.

MTT growth assay

To evaluate the growth capacity of SCLC cell lines, the cells were plated in RPMI + 10% FCS at 50000 cells per 0.2 ml in 96-well flat-bottomed microtitre trays (Costar, Cambridge, Massachusetts, U.S.A.) and cultured for different lengths of time. The cells number was evaluated by adding MTT [3(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St Louis, Missouri, U.S.A., 5 mg/ml] for 3 h to the culture and a final addition of dimethylsulphoxide (DMSO). The dye incorporated in the cells was determined by counting the optical density (O.D.) on an automated microplate reader with the spectrophotometer at 550 nm.

The doubling time was calculated as the ratio between the generation number and time. The generation number (n) was calculated as follows:

$$n = \frac{\log_{10}N - \log_{10}N_0}{\log_{10}2}$$

where N_0 is the number of cells at time T_0 and N is the number of cells at a considered time (T).

Soft agar assay

MBr1 cell growth inhibition was evaluated by the soft agar clonogenicity method. According to this method a single cell suspension of POVD cells (10^3 cells/well in 9.5-cm^2 area wells) was mixed with 0.3% agar containing purified MAb (concentration varying from 50 to 0.08 μg/ml), or medium as a control, and plated over a base layer of prehardened agar (1%) in six-well tissue culture trays (Costar). Colonies of > 50 cells were counted after 30 days.

Enzymatic treatments

POVD 10^6 cells were incubated with neuraminidase (Sigma) type X (1 U/ml) for 60 min at 37°C or with trypsin Flow (0.05%) for 5 min at 37°C. Following incubation the cells were washed twice with medium and then used for an indirect immunofluorescence assay.

Immunohistochemical tests

Indirect immunofluorescence on live cells, acetone-fixed cells and cryosections was performed using the purified antibody (10 μg/ml) and isothiocyanate-conjugated goat anti-mouse immunoglobulin (Ig) (Meloy Laboratories Inc., Springfield, Virginia, U.S.A.). The cells were incubated with the MAb for 30 min at 37°C, then washed three times with phosphate buffered saline/bovine serum albumin (PBS/BSA) and incubated with antimouse Ig for 30 min in ice. The cells were again washed three times, and resuspended in PBS/BSA. Fluorescein-labelled cells were analysed using a FACScan (Becton Dickinson).

Immunoperoxidase techniques were carried out as previously described [8, 12] on paraffin-embedded sections obtained from surgical specimens, using purified MBr1 antibody (10 μg/ml) and the avidin-biotin peroxidase complex (ABC) kit (Vector, Burlingham, California, U.S.A.).

Tumours were considered positive when more than 10% of the cells stained.

Tumour growth in athymic mice

Athymic CD1 mice (Charles River Laboratories, Wilmington, Massachusetts, U.S.A.) were injected subcutaneously with 5×10^6 SCLC cells (POVD, H128, H69, N592) and the tumour growth was evaluated after 30 days.

In tumour growth inhibition experiments, 24 h after the subcutaneous injection of POVD cells in nude mice, either 0.2 ml of ascitic fluid containing MBr1 or saline solution were injected intraperitoneally into the mice once daily for 10 days. Every day after the tumour injection the tumour size was measured in control and treated mice.

The data on tumorigenicity of MBr1-positive and -negative cell lines was investigated by means of the Mantel–Haenszel χ^2.

Immunoblotting procedure

The solubilisation of the POVD cells was carried out for 30 min in ice by adding 1 ml of 50 mmol/l Tris–HCl, pH 7.4, containing 1% antagosan (Behring, L'Aquila, Italy) and 1 mmol/l phenylmethylsulphonyl-fluoride (PMSF) (Sigma). The lysate was cleared by centrifugation and the soluble extract analysed by SDS–PAGE [13] in reducing conditions on a 5–15% polyacrylamide slab gel. Immunoblotting was carried out as described [14] with the exception that in the case of MBr1 the nitrocellulose paper (Metrical GN-6 Gelman, Ann Arbor, Michigan, U.S.A.) was directly reacted with 2.5×10^6 cpm/ml of ^{125}I-labelled MBr1 antibody.

RESULTS

Using immunohistochemical techniques, the reactivity of the MBr1 MAb was investigated either on frozen, paraffin-embedded sections or on cytospun cells obtained from a total of 161 SCLC specimens. As displayed in Fig. 1, the two groups of patients, divided according to the MBr1 reactivity on the tumour cells, showed statistically significant differences in their survival ($P < 0.001$), with a median survival time of 23 months for MBr1-negative patients versus 11 months for MBr1-positive patients.

The possibility that this shorter survival time was due to a greater aggressiveness of MBr1-positive tumours was pursued by analysing the growth capability of different SCLC cell lines, both *in vivo* and *in vitro*, in relation to marker expression.

CaMBr1-positive (H128, POVD) and CaMBr1-negative SCLC cells (H69 and N592), were tested for their capability to grow in nude mice. As shown in Table 1, a preferential growth of MBr1-positive cell lines was observed.

Similar results were obtained in *in vitro* studies. Indeed, Fig. 2 shows that the CaMBr1-positive POVD cell line grew faster (doubling time = 33 h) than the CaMBr1-negative H69 cell line (doubling time = 81 h). To further investigate the relevance of the CaMBr1 molecule expression in cell growth capability, the interference of MBr1 binding on the growth of CaMBr1-positive cells was evaluated. With this aim, MBr1 or an isotype-matched control MAb (MOv2) was added to the culture medium in a soft agar clonogenic assay performed with the POVD cells. As reported in Table 2, a dose-related inhibition of the colony formation was observed with MBr1, but not with the MOv2 MAb. Moreover, the clones grown in the presence of MBr1 were smaller than those grown in the control plates.

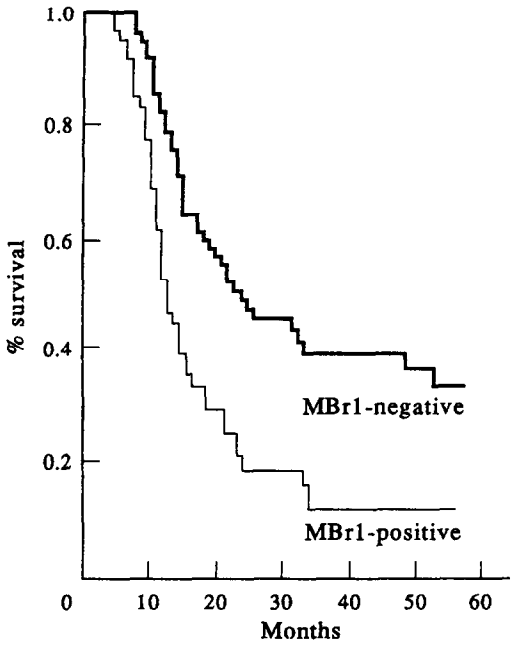

Fig. 1. Survival curves according to MBrl reactivity.

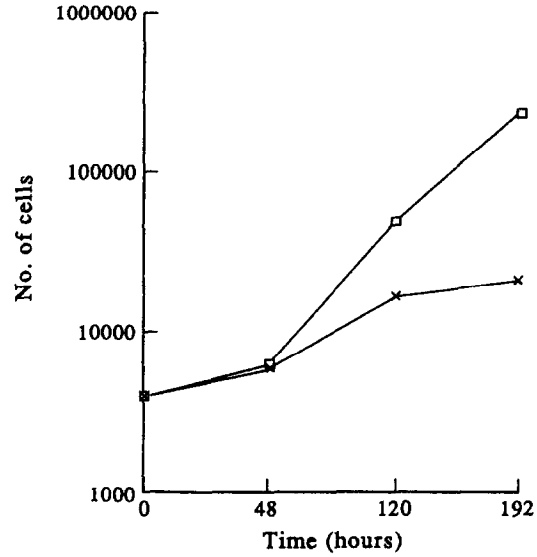

Fig. 2. Growth assay of SCLC cell lines: □ = POVD (MBrl-positive cells), × = H69 (MBrl-negative cells). The individual points referring to the number of cells at different times are the mean of five different experiments. The standard errors are not added in Fig. 2 since the values are too low to be reported.

Table 1. Relationship between CaMBrl expression and the growth capability in athymic mice of SCLC cells

Cell lines	MBrl reactivity	No. of mice with tumour growth/total of mice injected	%	*P*
H128	Positive	24/27	89	
POVD	Positive	9/10	90	< 0.01
H69	Negative	1/4	25	
N592	Negative	3/10	30	

P value for the Mantel–Haenszel χ^2 analysis.

Table 2. Inhibition of soft agar clonogenicity of the POVD SCLC cell line in the presence of MAb

MAb concentration (μg/ml)	% of clones	
	With MBrl	With MOv2
Control	100	100
0.08	78	100
0.40	88	94
2.00	71	81
10.00	52	100
50.00	32	90

CaMBrl expression was found to fluctuate on tumour cells grown *in vitro*. In the POVD cell line the percentage of MBrl-positive cells reached 80% at the time of the *in vivo* experiments of Table 1, and decreased after several months of *in vitro* passage to 34% positive cells reported in the enzyme sensitivity experiments (Table 3). This finding suggested an influence of the growth condition on the antigen expression, investigated in the cloning experiments.

The CaMBrl-negative cell line N592 was cloned by two different methods and the reactivity of the MBrl MAb was tested before, during and after cloning when the cells were allowed to grow again as a bulky culture. As shown in Table 4, N592 showed less than 10% positive cells before cloning. During the cloning with both methods the marker was upregulated and almost all the cells of every clone were positive. However, when these clones were all mixed together and grown as a bulky culture in flasks, a progressive decrease of the CaMBrl expression was found and the cell population returned to the initial 10% of positivity after 30 days.

Table 3. MAb reactivity by immunofluorescence on SCLC cells after enzymatic treatment

Target cells	MAb	Untreated		+ Neuraminidase		+ Trypsin	
		% Positive cell	Mean intensity	% Positive cell	Mean intensity	% Positive cell	Mean intensity
POVD	MBrl	34	173	60	288	12	102
	Leu7	36	153	34	166	N.T.	N.T.
N592	MBrl	39	88	62	96	5	49
	Leu7	43	84	53	80	N.T.	N.T.

N.T., not tested.

Table 4. MBr1 reactivity on N592 live cells after selection by cloning

Tested cells	% Clones with MBR1-positive cells	% MBR1-positive cells
Bulky culture	—	10
Soft agar clones	100	90
Limiting dilution clones	100	100
30 days bulky culture of soft agar clones	—	10

The inhibitory activity of the MBr1 MAb was also investigated *in vivo*. The MBr1 MAb was inoculated as ascitic fluid in nude mice bearing the POVD tumour. No differences in the tumour growth were observed between treated and untreated mice (Fig. 3).

Further experiments were carried out to study the biochemical nature of the CaMBr1 antigen on SCLC. CaMBr1-positive cell lines (POVD and N592 clones) were exposed to two different enzymes (trypsin and neuraminidase) and then tested for residual antigenic expression evaluated by immunofluorescence in flow cytometry. Both enzymatic treatments consistently changed the CaMBr1 expression on the treated cells: on both cell lines a decrease or an increase of the antigen were, respectively, observed after trypsin or neuraminidase treatment. However, neuraminidase increased the percentage of positive N592 cells from 39 to 62% without changing the fluorescence intensity, whereas on POVD both the intensity and number of positive cells were found to increase. On the same cells, the reactivity of the Leu7 MAb was found to be almost unchanged after neuraminidase treatment (Table 3).

In order to evaluate the molecular weight of the molecule, immunoblotting experiments were performed on POVD cell soluble extract. As shown in Fig. 4, a similar band of approximately 200 kD was detected when the blot was probed with the MBr1 and Leu7 MAbs (Fig. 4).

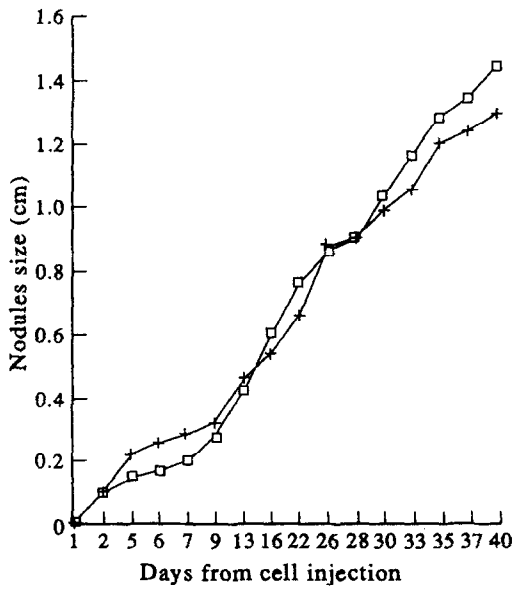

Fig. 3. POVD cell growth in nude mice in the presence of the MBr1 MAb: □ MBr1-treated mice, + untreated mice.

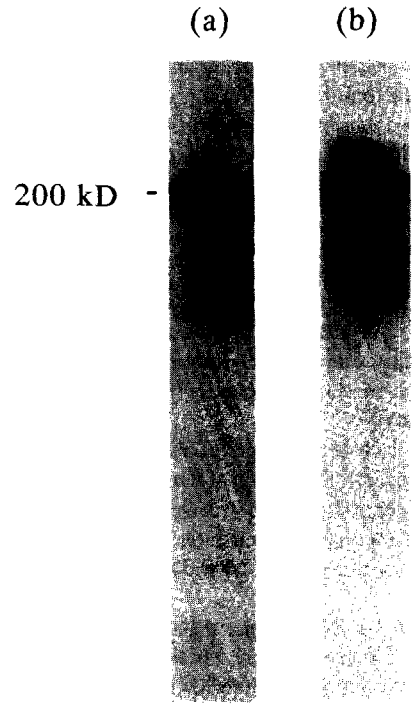

Fig. 4. Immunoreactivity of MAb MBr1 (Lane a) and Leu7 (Lane b) analysed by immunoblotting on a POVD soluble extract.

DISCUSSION

In the present study we confirm, on a larger number of cases, the previous findings regarding the correlation between the expression on the tumour of the epithelial antigen identified by the MBr1 MAb and the overall survival of SCLC patients. In particular, the "actuarial" survival of the CaMBr1-negative patients was greater than would be expected from a series of patients treated with conventional therapy, thus showing the prognosis impact of this marker. Indeed, a statistically significant association was found between the high expression of the marker and survival.

Recently, Bobrow *et al.* analysed a smaller series of 70 cases, selected as long-term and short-term survivors, with a panel of 17 MAb including the MBr1 MAb. In contrast to our present study and findings reported by others [3, 4], no independent prognostic significance was found for any of the tested markers [15]. These conflicting data could be due to differences in the size of the series, in the selection of the patients, in the sampling method, in the stage of the disease or in the treatment of the patient. In detail, the tested tissue specimens were different, being fibre-optic bronchoscopy specimens in one study instead of surgical specimens. Moreover, in our study the cases were not selected, whereas Bobrow's group selected the patients retrospectively, according to their survival time.

On the contrary, in keeping with the prognostic relevance of the CaMBr1 expression found by us, is the report on the MIA 15-5 MAb which recognises the same CaMBr1 saccharidic epitope. This MAb was found to identify adeno and squamous lung carcinomas with a poor prognosis and to inhibit *in vitro* tumour cell motility and *in vivo* metastasis [16, 17].

Our data suggest that the expression of this saccharidic epitope is also related to the growth capacity of the tumour cells. Indeed, the CaMBr1 expression was found to correlate with the growth of SCLC (Table 1).

The marker was up-modulated during clonal growth (Table

4), whereas its block with the relevant MAb inhibited the clonogenicity of the cells (Table 2).

The strong increase of CaMBr1 antigen expression on the N592 cell surface found after cloning could be explained by the selection of more aggressive and proliferating cells due to the limiting conditions of growth. The culture mass conditions resulted in a decrease in MBr1 reactivity of these MBr1-expressing cells, in agreement with the overwhelming number of cells with less aggressive capacity. This fluctuation in the percentage of MBr1-positive cells, due to culture conditions, explains the difference in MBr1 reactivity found in different experiments.

The relevance of the CaMBr1 antigen in tumour growth appears to be dose-dependent. Indeed, inhibition of *in vitro* clonal growth is directly related to the concentration of the blocking antibody.

The complete lack of effect of the treatment with MBr1 on the *in vivo* tumour growth is in contrast with a possible involvement of CaMBr1 in cell proliferation. However, the MAb could not have reached an adequate concentration in order to be active on the subcutaneous tumour, as already suggested in previous studies [18].

Concerning the biochemical nature of CaMBr1, we found that the antigen is trypsin-sensitive, which confirms the previous observation [5] that in SCLC the CaMBr1 epitope is carried by a glycoprotein structure, whereas in breast carcinomas both glycoprotein and glycolipid molecules were identified [19].

After neuraminidase treatment which cleaves sialic acid residues, a strong increase in MBr1 activity was found on two SCLC cell lines expressing the antigen. These results suggest the hypothesis that the sugar epitope recognised by the MBr1 MAb on the proteic bone of the molecule could be hidden by sialylation.

The fluctuation of MBr1 reactivity on SCLC cells grown in different conditions might, therefore, be explained either by changes in the expression of the whole molecule or by differences in the sialylation which change the CaMBr1 epitope availability for MAb binding.

In accordance, the prognostic significance of the MBr1 reactivity might be attributed to differences in the expression of the sialoglycoprotein or to changes in the sialylation of the membrane glycoprotein involved in the tumour aggressiveness. Therefore, in our study we found that the MBr1 MAb recognises a sialoglycoprotein, but the relevance of this CaMBr1 molecule in tumour progression is still unclear at present. However, we can suggest a role in the aggressiveness of the tumour, due to the presence of the glycoprotein or to the presence of sialic acid on the cell surface.

Variations in tumour cell invasive capabilities have been associated with differences in the amount of surface sialic acid and cell surface carbohydrate sialylation [20–22]. In lung tumours, a direct association between the decrease in surface sialic acid content and decreased invasive behaviour of the A549 adenocarcinoma cell line has been reported [23]. Therefore, the aggressiveness of CaMBr1-positive tumours might be related to the expression of a particular sialo-glycoprotein, in which the sialylation could also interfere with the function.

Many polysialoglycoproteins have been shown to be expressed on SCLC cells. The most important ones belong to the homophilic adhesion molecule family including L1, J1, N-CAM and MAG [24, 25] recognised by different MAbs such as Leu19 and Leu7. We cannot say at present whether the CaMBr1 epitope is carried by one of these molecules. However, the similar molecular weight of the molecule (200 kD) detected by immunoblotting with the MBr1 MAb and the Leu7 MAb, which recognises the myelin-associated glycoprotein [26–28], could suggest that on this cell line the same molecule bears the epitopes recognised by the two MAbs.

In a previous study [5], MBr1 was found to recognise a 145 kD and a 270 kD molecule in soluble extracts from SCLC biopsies. The molecular weight of the CaMBr1 molecule was found to be different on SCLC according to the tested material: two molecules of 145 and 270 kD molecular weight were found on surgical specimens, and a 200 kD molecular weight was obtained from established cell lines. This discrepancy could be explained by a different level of sialylation and glycosylation between *in vitro* cells and *in vivo* tumours.

In conclusion, the MBr1 MAb seems to identify a molecule which is relevant for tumour aggressiveness. Further studies are needed to clarify whether the role of the CaMBr1 marker in tumour aggressiveness is due to the expression of the epitope itself, to the molecule/s carrying the epitope or to other structures which interfere with the epitope recognition, such as sialic acid.

Moreover, with controlled clinical studies we will be able to confirm the prognostic value of the CaMBr1 marker.

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Serum Beta-lipoprotein, Serum Cholesterol and Quetelet's Index as Predictors for Survival of Breast Cancer Patients

Sven Törnberg and John Carstensen

We studied the survival of breast cancer patients in relation to serum cholesterol level, serum beta-lipoprotein level (BLP) and being overweight among women having breast cancer diagnosed during a follow-up period of 20 years. A cohort of 46 570 women attended a general health screening including examination of serum lipid levels, height and weight during 1963–1965. Of these, 1170 women developing breast cancer; 196 were below the age of 50 and 974 were above 50 years of age. 66 of the younger women, and 341 of the older women were reported to have died of breast cancer. A correlation was found between high serum BLP and decreased survival of breast cancer patients < 50 years of age. For women ≥ 60 years of age, BLP was positively correlated to breast cancer survival. No correlation was found between serum cholesterol level and breast cancer survival in any age group. Increasing obesity was statistically significantly correlated to decreased survival with breast cancer. The latter findings were in accordance with other studies which have shown being overweight as a risk factor for breast cancer. As for the relationships between ischaemic heart disease and serum lipid levels, in studies of cancer risks in relation to serum cholesterol level, the different fractions of cholesterol seem to be of importance.
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INTRODUCTION

SUGGESTIONS HAVE been made that factors related to breast cancer development may also interfere with its prognosis [1–3]. Apparently, being overweight is associated with an increased breast cancer risk in postmenopausal women and decreased risk in premenopausal women [4]. Being overweight also seems to have an unfavourable impact on breast cancer survival, which is most pronounced among postmenopausal women [5–15]. High serum cholesterol levels have, in two studies, been correlated to decreased breast cancer survival [9, 15]. However, in studies on serum cholesterol levels and risk of breast cancer, most researchers have failed to demonstrate a positive relationship [16–20].

Serum beta-lipoprotein (BLP) is the electrophoretical correspondent to the ultracentrifugally measured low-density lipoprotein (LDL). BLP has been more strongly correlated to dietary