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Prognostic Value of Immunohistochemical Estimation of CD24 and Ki67 Expression in Cisplatin and Paclitaxel Treated Ovarian Carcinoma Patients*

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CD24 is a small membranous protein which may participate in invasion of tumor cells. Present study aimed at evaluation of prognostic significance linked to immunohistochemical demonstration of CD24 expression and the proliferation index, Ki67 expression in ovarian cancers. The immunohistochemical reactions with monoclonal CD24- and Ki67-specific antibodies were performed in paraffin sections originating from 30 patients with ovarian cancer treated using cisplatin and paclitaxel. Results of the reactions and analysis of the clinical course of the patients were subjected to statistical analysis. Cases with cytoplasmic-membranous expression of CD24 (CD24c-m) were found to exhibit significantly shorter overall survival time (*P*=0.0002) and progression-free period (*P*=0.0005). Cases with membranous expression of CD24 (CD24m) manifested a longer overall survival time (*P*=0.022). No relationship was disclosed between expression of Ki67 on the one hand and survival time and CD24 expression on the other. As documented using chi square test, expression of CD24c-m predisposed to relapses (*P*=0.012), progression (*P*=0.0362) and to death (*P*=0.0034). Deaths were encountered significantly less frequently in cases with CD24m expression (*P*=0.0465). The studies demonstrated that CD24c-m represented a strongly unfavorable prognostic indicator. The antigen represents an interesting target in the search for novel therapeutic methods. The more aggressive course of cases with CD24c-m expression was not linked to more intense proliferation of the tumor cells.

Introduction

Ovarian cancers are the most frequent gynecological tumors of female sex. Due to their location and, linked to it, late diagnosis as well as due to the aggressive course of the disease, therapy of ovarian cancer seldom leads to cure. Despite introduction of new therapeutic modalities, proportion of 5-year survival for all clinical stages of the cancer in the recent 20 years did not exceed 40%. Seventy-five % of all ovarian cancer cases are diagnosed at the III or IV FIGO stage. In the groups only about 20% of the patients survive 5 years [7]. Therefore, in several centers intense search continues for new prognostic indices, which would permit intensification of therapy in high risk cases and which could provide target for novel therapeutic modalities.

Membranous CD (cluster of differentiation) proteins were originally described as specific markers of various subtypes of lymphoid cells. At present, expression of individual CD antigens provides grounds for classification of hematological tumors [6]. In recent years, some of the CD antigens have been demonstrated also on cells of epithelial tumors and a proportion of them have manifested a significant relationship to the clinical course of the disease [12, 13, 15].

CD24 is a small membranous protein, typical for lymphocytes B [14]. Expression of the protein was demonstrated also in the course of the development of pancreas [2], brain [19] and in regenerating muscles [3]. The expression was noted also in several types of neoplasms [9–11]. In 2002 we described CD24 expression in ovarian cancers [8]. In studies

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performed on 69 cases of ovarian cancer of variable histological type, advancement stage and applied therapy CD24 expression was demonstrated to be an unfavorable prognostic factor. The prognostic significance of CD24 expression was explained by participation of the protein in the process of neoplastic invasion. The study group of tumors was not uniform in respect to histogenesis as well as grade, stage or applied therapy. Due to low numerical force of the group no multivariate analysis was performed. Prognostic value of CD24 requires confirmation on larger groups of patients or on uniform groups. Until now, relationships between CD24 expression and exponents of tumor dynamics, such as proliferation, have not been examined.

Ki67 represents a labile, non-histone protein present in the cell nucleus. The protein is present in cells undergoing a mitotic cycle. On the other hand its presence could not have been detected in cells entering the G0 phase of the cell cycle [17]. Expression of Ki67 used to be estimated in order to determine the proportion of dividing cells. An unfavorable prognostic significance of elevated Ki67 expression was demonstrated in biopsies of ovarian cancer, originating from primary laparotomies [5, 20].

The present study aimed at examining prognostic value of CD24 expression and of its relation to proliferation intensity measured by Ki67 expression in a uniform group of patients with ovarian cancer, treated with cisplatin and paclitaxel.

Material and Methods

Patients

Immunohistochemical examination was performed retrospectively on tissue samples taken for routine diagnostic purposes. Thirty patients operated in 1999–2002 due to ovarian carcinoma in the Department of Gynaecology and Obstetrics, University Medical School in Poznań were included in the study. The cases were selected based on availability of tissue and were not stratified for known preoperative or pathological prognostic factors. The study was approved by an Institutional Review Board (IRB) and the patients gave their informed consent before their inclusion into the study. Following the surgery all the patients were subjected to chemotherapy using cisplatin and paclitaxel (Table 1). The patients were monitored by periodic medical check-ups, CA-125 serum levels, ultrasonographic and radiological examinations.

Tissue samples were fixed in 10% buffered formalin and embedded in paraffin. In each case, hematoxylin and eosin stained preparations were subjected to histopathological evaluation by two pathologists. The stage of the tumors was assessed according to the International Federation of Gynaecology and Obstetrics [21]. Tumors were graded according to the Silverberg grading system [18].

TABLE 1
Patient and tumor characteristics

Characteristics	No. (%)
All patients	30 (100)
Age (mean 51.3)	
≤50	13 (43)
>50–60	13 (43)
>60	4 (14)
Grade	
1	3 (10)
2	13 (43)
3	14 (47)
FIGO	
II	1 (3)
III	29 (97)
Histology	
Serous	27 (90)
Other	3 (10)
Clinical response	
Complete response	13 (43)
Stable disease	5 (17)
Progressive disease	12 (40)
Chemotherapy	
Cisplatin/Paclitaxel	30 (100)

Immunohistochemistry

Formalin-fixed, paraffin embedded tissue was freshly cut (4 μm). The sections were mounted on Superfrost slides (Menzel Gläser, Germany), dewaxed with xylene, and gradually hydrated. Activity of endogenous peroxidase was blocked by 5-min exposure to 3% H_2O_2. All the sections studied were boiled in the Antigen Retrieval Solution (DakoCytomation, Poland). Then, immunohistochemical reactions were performed using the following antibodies:

1. mouse monoclonal antibodies (clone SN3) against CD24 (DakoCytomation, Poland) (dilution 1:100);
2. mouse monoclonal antibodies (clone MIB-1) detecting Ki67 (DakoCytomation, Poland) (dilution 1:100).

The antibodies were diluted in the Antibody Diluent, Background Reducing (DakoCytomation, Poland). Tested sections were incubated with antibodies for 1 h at room temperature. Subsequent incubations involved biotinylated antibodies (15 min, room temperature) and streptavidin-biotinylated peroxidase complex (15 min, room temperature) (LSAB+, HRP, DakoCytomation, Poland). NovaRed (Vector Laboratories, UK) was used as a chromogen (10 min, at room temperature). All the sections were counterstained with Meyer's hematoxylin. On every case, control reactions were included, in which specific antibody was substituted by the Primary Mouse Negative Control (DakoCytomation, Poland).

Intensity of immunohistochemical reactions was evaluated independently by two pathologists. In equivocal cases, the preparation was re-evaluated in common. In evaluation of CD24 expression intensity the previously described scale was employed [9–11], which took into account location of the reaction: membranous (CD24m) or cytoplasmic-membranous (CD24c-m). Cases with no CD24 expression or the expression in less than 10% of the cells were denoted by 0 while cases with presence of CD24 in cancer cells were marked by 1. In the case of Ki67 percentage of cells manifesting the reaction was evaluated.

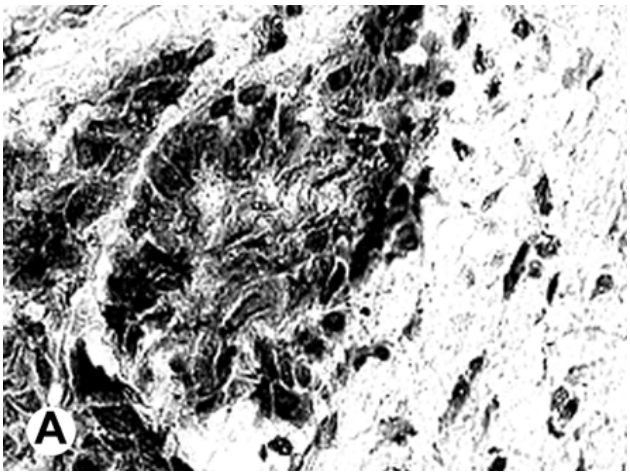
Statistical analysis

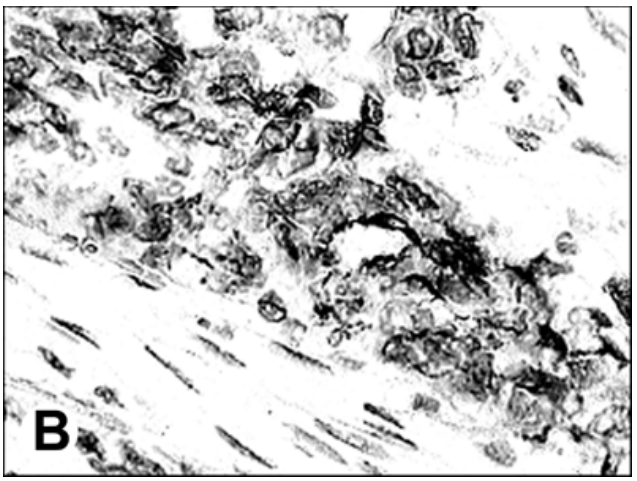
Statistical analysis of the results took advantage of Statistica 98 PL software (Statsoft, Poland). Relations between individual variables were tested using the chi square test and the ANOVA rank test of Kruskal-Wallis. Kaplan-Meier's statistics and log-rank tests were performed using SPSS software (release 10.0; SPSS Inc., Chicago, IL, USA) to estimate significance of differences in survival times. The length of progression-free survival was defined as the time between the primary surgical treatment and diagnosis of a recurrent tumor or death.

Results

The immunohistochemical reactions yielded the following pattern:

1. staining for CD24 protein resulted in reactions of a cytoplasmic-membranous localization (CD24c-m) (Fig. 1A) or membranous localization (CD24m) (Fig. 1B). CD24c-m was demonstrated in 14 (47%) cases and CD24m in 11 (37%) cases. Only in 5 (17%) cases expression of CD24 could not be demonstrated;





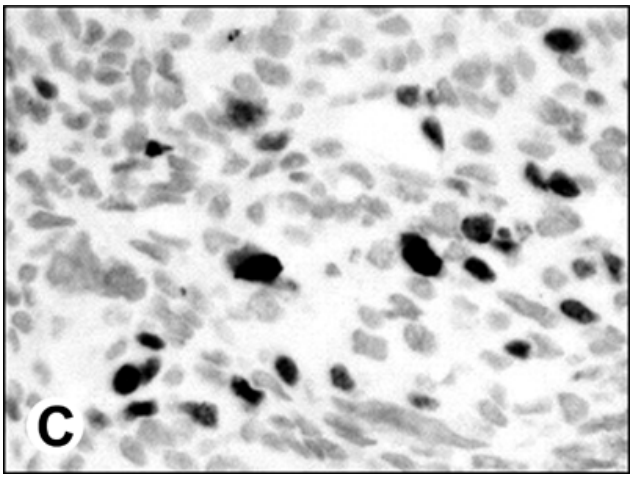


Fig. 1. Immunohistochemical localization of: A. Cytoplasmic-membranous expression of CD24; B. Membranous expression of CD24; C. Ki67 expression in ovarian carcinoma sections. Orig. magn. 400×.

2. staining for Ki67 resulted in color reaction of a nuclear localization. In individual cases the reaction developed in a variable proportion of cancer cells (Fig. 1C). Mean proportion of cells manifesting the reaction amounted to 53% ± 23 SD.

At the first step of statistical analysis the chi square test was used. Relations were examined between expression of CD24 and Ki67 on the one hand and extent of differentiation of the tumors, development of relapses, response to therapy and death of the patients. The following significant relationships were disclosed:

1. relapses developed with a significantly higher frequency in cases with CD24c-m expression (Table 2);
2. in cases with CD24c-m expression progress of the neoplastic disease was noted with a significantly higher frequency than full remission or stable disease (Table 2);
3. death was more frequent in cases with CD24c-m expression and significantly less frequent in cases with CD24m expression (Table 2).

No relationship could be disclosed between expression of the proteins and the extent of tumor differentiation (Table 2).

The ANOVA rank test of Kruskal-Wallis was used in order to examine a relationship between expression of CD24c-m or CD24m and intensity of proliferation estimated by Ki67. The analysis failed to disclose any relationships ($P>0.05$).

Using the analysis of Kaplan-Meier the complete survival time and the period with no progression were compared between groups demonstrating (1) and those not demonstrating (0) expression of CD24. In the case of Ki67 the study material was subdivided into subgroups with lower than average and that with the higher than average expression. The so prepared calculations demonstrated that:

1. cases with CD24c-m expression exhibited a significantly shorter total survival time and progression-free survival time (Figs. 2A and 2B);
2. expression of CD24m predisposed the patient to a longer total survival time (Figs. 2C and 2D);
3. expression of Ki67 was not related to total survival time or relapse-free survival time ($P>0.05$).

TABLE 2
Relationships between pathological and clinical variables of the study patients and expression of CD24 and Ki67 (chi square test)

Studied protein	Grade	Relapse	Response	Death
CD24c-m	0.0938	**0.0012**	**0.0362**	**0.0034**
CD24m	0.7755	0.2871	0.2399	**0.0465**
Ki67	0.4129	0.0636	0.1767	0.3613

Discussion

Effect of CD24 on the course of neoplastic disease was explained by the phenomenon originally described in 1997 by Aigner et al. [1]. In their studies on P-selectin present on endothelial cell membranes and participating in the rolling phenomenon the authors demonstrated that CD24 represented its ligand. CD24 interacted with the endothelial P-selectin, which facilitated intra- or extravasation of the cell. Thus, CD24 may participate in intravasation of tumor cells and, consequently, with formation of metastases. The hypothesis was corroborated by Schindelmann et al. [16], who demonstrated that CD24 expression in mammary cancer cells strongly correlated with their augmented capacity to form metastases. The unfavorable prognostic significance of CD24 expression was demonstrated in various tumors, including ovarian, breast and prostate cancers [8, 9, 11].

In the present group of ovarian cancer patients uniform in respect to the applied therapy (postoperatively all the patients received cisplatin and paclitaxel) and in respect to advancement stage (97% FIGO III, 3% FIGO II stage) prognostic value of CD24 expression was examined using immunohistochemical reactions. Cases with CD24c-m expression were found to exhibit a significantly shorter total survival time and progression-free time. Expression of CD24c-m predisposed also to more frequent relapses of the disease, to progression of the process following chemotherapy and to death due to the neoplastic process. Considering the fact that no relationship could be documented between expression of CD24c-m and the proliferation exponent, Ki67, the more aggressive course of cases with CD24c-m expression might be related to their higher invasive capacity.

In our study we have demonstrated the up to now not disclosed phenomenon of favorable prognostic significance linked to CD24m expression. Manifestation of CD24 of membranous location has been typical for cases with longer total survival time. In analysis of the phenomenon one should keep in mind that as many as 14 out of 19 (74%) cases negative for CD24m have been in parallel positive for CD24c-m. It should also be noted that statistical significance of the relation between CD24c-m and the survival time has been much higher than that for respective relationship between CD24m and the survival time ($P=0.0002$ and $P=0.0222$, respectively). In earlier studies [8–11] we have demonstrated also that cytoplasmic location of CD24 is prognostically particularly unfavorable. The cytoplasmic-membranous location results most probably from intensified expression of CD24. Presence

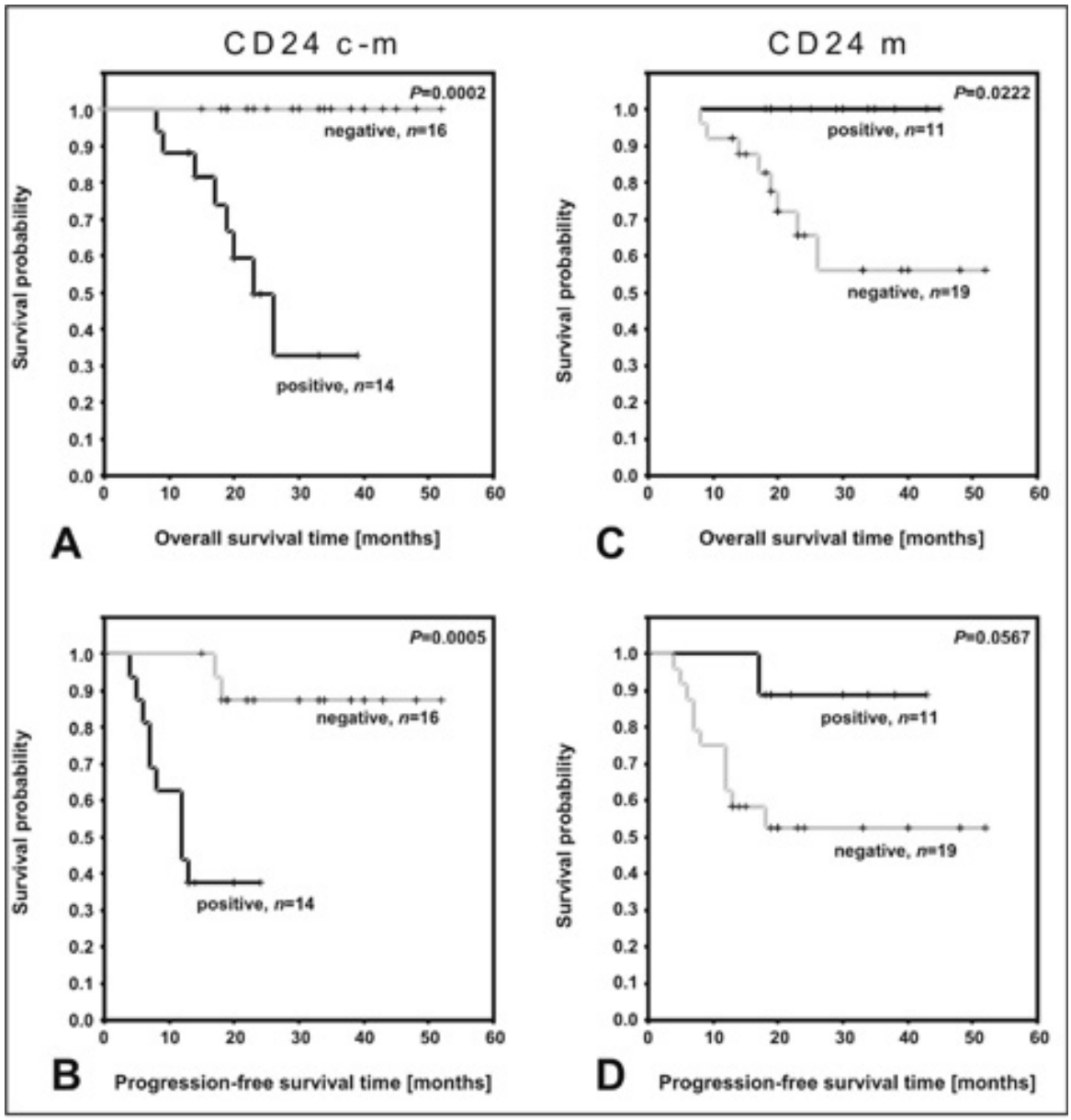


Fig. 2. Relationship between CD24c-m expression and total survival time (A) or relapse-free survival time (B), between CD24m expression and total survival time (C) or relapse-free survival time (D). Cases with CD24c-m expression demonstrated shorter total survival time (P=0.0021) and relapse-free survival time (P=0.0013). Cases demonstrating expression of CD24m showed longer total survival time (P=0.0222) (Kaplan-Meier's analysis).

of the protein in the cytoplasm may reflect membrane protein turnover and transport of proteins from endoplasmic reticulum to the cell membrane.

Prognostic significance of Ki67 expression in ovarian cancer is not unequivocal. Some authors have demonstrated an unfavorable prognostic significance of higher Ki67 expression [5, 20] but others could not have detected such a relationship [4]. In our study we have not been able to demonstrate relationships between Ki67 expression and studied clinical and pathological variables.

Summing up, in our study we have demonstrated high prognostic value of immunohistochemical estimation of CD24 expression in ovarian cancers. No relationships could be documented between CD24 expression and intensity of proliferation, measured using Ki67 protein. CD24c-m represents an interesting unfavorable prognostic index, the expression of which in ovarian cancers should be regarded to indicate need for implementation of a more intense therapy. CD24 provides also an interesting target for studies on novel therapeutic modalities.

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