Pergamon

European Journal of Cancer Vol. 31A, No. 10, pp. 1706–1709, 1995
Elsevier Science Ltd
Printed in Great Britain.
0959–8049/95 $9.50+0.00

0959-8049(95)00353-3

Original Paper

Prognostic Value of CD44 Splice Variants in Human Stage III Cervical Cancer

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The expression of specific cell adhesion molecule CD44 isoforms (splice variants) has been shown to be associated with poor prognosis in human malignancies, such as breast cancer. We used three different variant exon sequence-specific murine monoclonal antibodies to epitopes encoded by exons v5, v6 or v7–v8 of human variant CD44, to study the expression of CD44 splice variants by immunohistochemistry in human stage III cervical cancer. We investigated 40 pretreatment punch biopsies of cervical cancer FIGO stage III. CD44 splice variants CD44v5, CD44v6 and CD44v7–8 were detected by means of immunohistochemistry in 90%, 55% and 25%, respectively. CD44 epitopes encoded by exon v5 were not correlated with prognosis. Expression of CD44 splice variants containing epitopes encoded by exon v6 were correlated with significantly poorer prognosis (Mantel test, $P = 0.008$). Five-year survival rates with or without CD44v6 expression were 20% versus 71%, respectively. Expression of CD44v7–8 was also correlated with significantly poorer overall survival (Mantel test, $P = 0.02$). Expression of CD44 splice variants containing epitopes encoded by exons v7–v8 and especially exon v6 is associated with significantly poorer prognosis in stage III cervical cancer patients.

Key words: uterine cervix, neoplasm, adhesion molecules, prognosis
Eur J Cancer, Vol. 31A, No. 10, pp. 1706–1709, 1995

INTRODUCTION

NEOADJUVANT AND concomitant multimodality therapies have been studied in patients with advanced cervical cancer [1–3]. New treatment protocols in advanced stages of cervical cancer, including radiotherapy following initial chemotherapy or a combination of chemotherapy, surgery and radiotherapy, are under evaluation [3, 4]. In this discussion of new treatment modalities in advanced stages of cervical cancer, assessment of prognosis is important in the selection of patients for new treatment protocols.

In cases without primary surgery, evaluation of prognostic parameters is limited to histological examination of biopsies, thus providing histological type of the tumour and grading. Although imaging methods provide sufficient tools for staging, other major prognostic factors cannot be evaluated as accurately as in surgically treated cases. Without lymph node sampling, lymph node status, which has been shown to be of prognostic importance [5, 6], cannot be evaluated accurately. Therefore, prognostic parameters associated with lymph node metastasis could be helpful in these cases where no surgical specimen of the pelvic nodes can be examined.

The CD44 family of transmembrane receptor molecules is derived from a single gene located on chromosome 11. By the mechanism of "messenger RNA alternative splicing", numerous isoforms of the CD44 protein are produced [7–9]. The expression of specific CD44 isoforms (splice variants) has been shown to be associated with metastasis and poor prognosis in human malignancies, such as breast cancer, colorectal cancer and gastrointestinal lymphoma [10–12].

Few data concerning the expression of CD44 splice variants in human cervical cancer exist [13], and no data concerning the prognostic value of CD44 splice variants have yet been published. Therefore, we examined the expression of CD44 splice variants in probe excisions of the primary tumour in a group of FIGO stage III cervical carcinoma patients undergoing radiotherapy.

MATERIALS AND METHODS

We investigated 40 randomly selected pretreatment punch biopsies of cervical cancer FIGO stage III. Staging was done by inspection, palpation and rectal sonography [14]. The mean age of the patients was 60.5 (S.E. ± 18.9) years. Thirty of the tumours were squamous cell carcinomas (10 keratinising, 20

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Revised 14 Feb. 1995; accepted 20 Feb. 1995.

non-keratinising), 6 were small cell carcinomas and 4 adenocarcinomas. All patients underwent a combined standardised primary irradiation therapy between 1985 and 1989. Follow-up examinations were performed at 3 month intervals, including vaginorectal palpation, ultrasound and serum tumour marker examination (Squamous Cell Carcinoma Antigen). Computed tomography of the pelvis was performed every 6 months. If any doubtful findings were found or serology showed elevated levels, additional imaging examinations were performed.

For CD44 splice variant analysis, we used three different variant exon sequence-specific murine monoclonal antibodies to epitopes encoded by exon v5, exon v6 or exon v7–v8 of human variant CD44, respectively [15].

Immunohistochemistry

We prepared all sections from routine formalin fixed paraffin embedded specimens. Sections were deparaffinised in xylene and rehydrated in a graded alcohol series (100%–70%). To recover antigenicity, we used the "Antigen Retrieval System" (Bio Genex, San Ramon, California, U.S.A.) twice for 20 min in the microwave at 600 W power (HM 146, Elektra Bregenz, Schwaz, Austria) and then rinsed sections in 10 mM BS (pH 7.6). Three different primary antibodies to different epitopes encoded by human CD44 splice variants were used [15, 16]: the first was the monoclonal antibody to an epitope encoded by exon v5 (CD44v5, Clone VVF-8, Bender Co., Vienna, Austria) [15], the second antibody was to an epitope encoded by exon v6 (CD44v6, Clone VFF-7, Bender Co., Vienna, Austria), and the third antibody was to an epitope encoded by exon v7–v8 (CD44v7–8, Clone VFF-17, Bender Co., Vienna, Austria). The primary antibody was diluted in serum/PBS (phosphate-buffered saline) and the sections were incubated for 60 min and then incubated for a further 30 min with biotinylated anti-mouse and anti-rabbit link-antibody (DAKO LSAB 2 Kit; DAKO, Carpinteria, California, U.S.A.). After rinsing in PBS, the sections were coated with streptavidin conjugated to alkaline phosphatase for 10 min. The sections were rinsed in PBS, incubated with Fast Red chromogen (naphthol phosphate substrate in Tris buffer, Fast Red chromogen tablets 5 mg, BioGenex, San Ramon, California, U.S.A.) and then washed with distilled water. The sections were finally counterstained with haematoxylin and mounted. We interpreted strong and/or widespread staining at positive, weak and focal staining as negative.

Positive control. The positive control slide was prepared from epidermal tissue, known to contain the antigen (Figure 1). In the positive control tissue, all monoclonal antibodies stained similarly.

Negative control. The negative control slide was prepared from the same tissue block as the specimen. However, instead of using a primary antibody we used a normal, non- immune serum supernatant from the same source as the primary antibody.

Statistics

Chi-square test was used where appropriate. To compare nominal variables of two groups, the Student's *t*-test was used. Results were analysed for the endpoint of overall survival. Overall survival was defined as the period between the date of primary surgery and death. Survival times of patients still alive were censored with the last follow-up date (last follow-up date or date of death), respectively. Survival probabilities were calculated by the product limit method of Kaplan and Meier [17]. Differences between groups were tested according to Mantel [18]. All *P*-values are results of two-sided tests. The BMDP statistical software system (BMDP Statistical Software, Inc., Los Angeles, California, U.S.A., 1990) was used for the calculations.

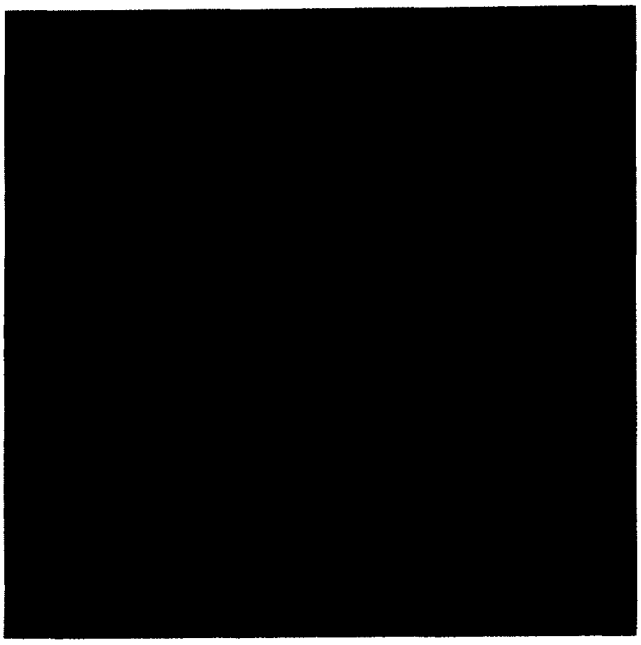

Figure 1. Expression of CD44 containing epitope v6 in normal skin epithelium (positive control) with staining of cell membranes, magnification × 140, counterstain, haematoxylin.

RESULTS

In accordance with the findings of Dall and associates on frozen sections [13] we found membranous staining with the antibodies against epitopes encoded by CD44v5 (Figure 2) and CD44v6 in the basal and parabasal cells but no staining with CD44v7–8 in normal cervical epithelium.

CD44 splice variants containing CD44v5, CD44v6 and CD44v7–8 were detected by means of immunohistochemistry in 90% (n = 36), 55% (n = 22) and 25% (n = 10) of the 40 tumour samples, respectively (Table 1). We found homogeneous staining in tumours that were considered positive for CD44 expression, and Figure 3 shows expression of CD44v6 at the cell membranes of cervical carcinoma. Only CD44v6 and CD44v7–8 showed a significant co-expression, CD44v6 was positive in 9 of 10 (90%) cases when CD44v7–8 was positive and only 13 of 30 (43%) cases when CD44v7–8 was negative (Chi-square test, P = 0.01). We found no correlation between expression of any of the splice variants and age. Expression of splice variants in different types of cervical carcinomas is shown in Table 1.

CD44 epitopes encoded by exon v5 were not correlated with prognosis (Mantel test, P = n.s.). Expression of CD44 splice variants containing epitopes encoded by exon v6 were correlated with significantly poorer prognosis (Mantel test, P = 0.008, Figure 4). Five year survival rates with or without CD44v6 expression were 20% versus 71%, respectively. Expression of CD44v7–8 was also correlated with significantly poorer overall survival (Mantel test, P = 0.02). Patients with tumours with and without expression of CD44v7–8 had 5 year survival rates of 30% and 48%, repectively.

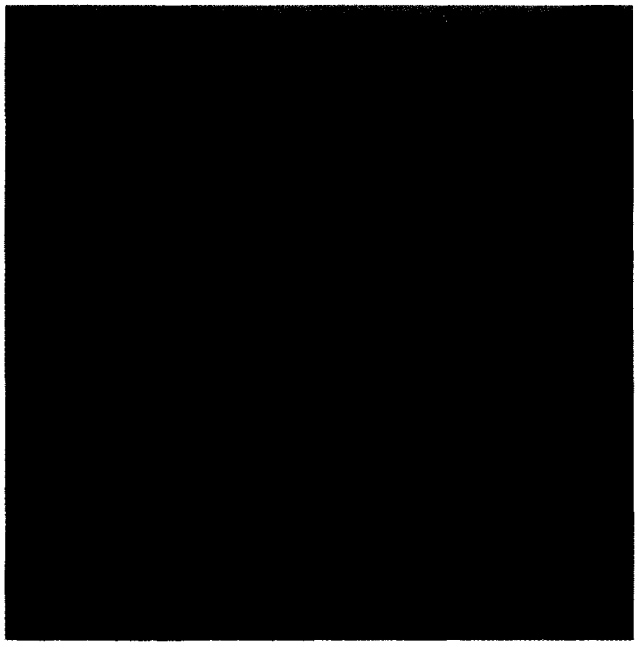

Figure 2. Expression of CD44 containing epitope v5 in normal cervical epithelium with staining of cell membranes of basal and parabasal cells, cervical stroma cells are negative, magnification × 80, counterstain, haematoxylin.

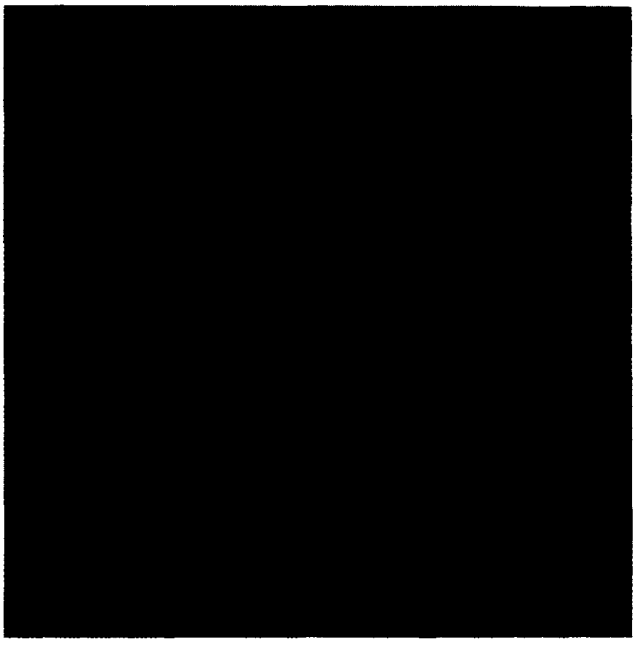

Figure 3. Expression of CD44 containing epitope v6 in cervical carcinoma tissue, cervical stroma cells are negative, magnification × 200, counterstain, haematoxylin.

Table 1. Expression of CD44 splice variants in tumour types

	Number of positive tumours		
	CD44v5	CD44v6	CD44v7–8
Squamous cell carcinoma			
-keratinising (n = 10)	10	8	1
non-keratinising (n = 20)	19	12	8
Small cell carcinoma (n = 6)	5	1	0
Adenocarcinoma (n = 4)	2	1	1
Total (n = 40)	36	22	10

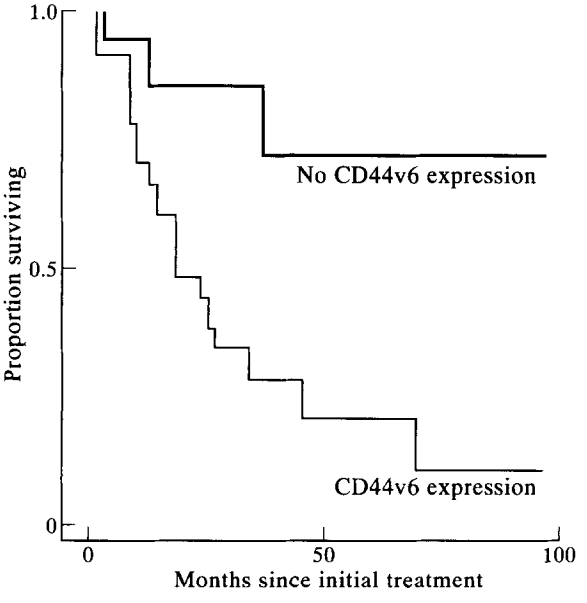

Figure 4. Overall survival of patients with cervical cancer stage III tumours, with or without expression of splice variant CD44v6.

DISCUSSION

Currently, the only published data concerning immunohistochemically detected expression of CD44 splice variants in cervical cancer have been provided by Dall and colleagues [13]. In this study, 16 cervical cancer tumours, stages IB–IIIB, were investigated for the expression of CD44 splice variants epitopes encoded by exons v7 and v8. Fifteen of 16 tumours stained strongly for CD44v7–8 when snap-frozen tissue samples were used for immunohistochemistry. Using the same monoclonal antibody against the epitope encoded by the CD44v7–8 on paraffin embedded tissue samples, we found positive staining in only 9 of 40 FIGO stage III tumours. This difference could be due to the use of different specimens (snap-frozen versus paraffin embedded, different tumour stages). However, we found a high percentage of tumours (36/40) expressed splice variants CD44v5. Currently, there are no published data available to compare the expression of splice variants of CD44 expressing epitopes encoded by exon v5 or exon v6.

Evaluation of prognosis is important when new treatment protocols for advanced cervical cancer stages are investigated [1–4]. Expression of specific CD44 isoforms has been shown to be associated with poorer prognosis in other human malignancies, such as breast cancer, colorectal cancer and gastrointestinal lymphoma [10–12]. In this study of the three investigated CD44 splice variants, expression of CD44v6 (Figure 1) and CD44v7–8 was significantly correlated with poorer overall survival in stage III cervical cancer. Splice variants of CD44 (exons V4–V7) have been shown to confer metastatic behaviour to adenocarcinoma cell lines in a rat carcinoma model [19, 20]. Unfortunately, this preliminary study cannot clarify if the poorer prognosis is associated with more advanced metastatic spread of the tumour, since pretreatment staging and lymph node status were not available for all patients.

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Acknowledgement—Supported by the Research Grant of the Major of Vienna (No. 1045 (to Dr Kainz)).