Invasive cribriform breast carcinomas in patients with grade 1 and stage IIA (T2 N0 M0) breast cancer strongly express the v3 and v6, but not the v4 isoforms of the metastatic marker CD44

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Patients with breast carcinomas of the invasive cribriform (IC) histological type often have excellent prognosis. Nevertheless, prognostic markers such as CD44v3, v4, and v6 isoforms have never been evaluated in this histological type.

Cases seen between 1996 and 2006 at two major public hospitals in Kuwait were reviewed. We selected the cases which still had enough tissue in the paraffin blocks, had pure rather than mixed typical histological type, did not receive hormonal or any other type of therapy prior to or at the time the tumor was excised, and which were grade 1, and stage IIA (T2, N0, M0). This is to control for confounding factors that could affect the degree of tumor expression of the above isoforms. Sections were immunostained using a highly sensitive peroxidase-anti-peroxidase kit, and scoring of immunostaining was performed in a semi-quantitative manner as established in the literature.

An extensive expression of the CD44v3 and v6 isoforms was seen in 83.3% of the IC tumours, while 83.3% lacked the expression of the v4 isoform. A significant association between the histological type and degree of expression of CD44 isoforms was found only with the v3 isoform. The degree of expression of the v3 isoform was significantly different in the IC tumors as compared to the papillary, invasive lobular, and invasive ductal (NOS) ones. There was a significant negative correlation (r_s = - 0.201) between the expression of the v4 and v6 isoforms.

IC tumors seem to have a strong expression of the prognostic markers v3 and v6 isoforms of the transmembrane molecule CD44, and to lack the expression of the v4 isoform.

Key words: Kuwait, invasive cribriform breast carcinoma, CD44v3, v4, and v6 isoforms.

Human breast carcinomas of the invasive cribriform (IC) histological type often have a remarkably favourable outcome, with the 10-year overall survival being between 90% and 100% [1–3]. These carcinomas account for up to 3.5% of all breast carcinomas, and the mean age of the patients presenting with such a tumor is between 53 and 58 years [1, 2, 4]. Clinically these tumors are often of a very small size, histologically are arranged as invasive, often angulated islands, in which well-defined spaces are formed by arches of the cells. Apical snouts are regular features, and the tumor cells are often small, with a low to moderate nuclear pleomorphism. It is rare to find mitoses in these cells, and a prominent reactive-appearing fibroblastic stroma is often seen in these tumors. The incidence of axillary lymph node metastases in IC carcinomas can be as much as 14.3%, and the tumors are 100% and 69% positive for oestrogen and progesterone receptors, respectively [1, 2].

The expression pattern of CD44 in breast cancer is still not clear, and its role as a reliable prognostic marker is still controversial [5–10]. CD44 is an integral transmembrane molecule that is predominantly located extracellularly. It was originally described as a lymphocyte-homing receptor which enables lymphocytes to adhere to high endothelial venules [11]. The carboxyterminal end is intracellular, while the extracellular part consists of the middle variable and the aminoterminal (hyaluronan or HA binding) domains. The variable domain is where differing isoforms express their characteristic variant protein, encoded by the variant exons v2-v10. The most prolific isoform of CD44 is the standard isoform being referred to as CD44s. Variant CD44 can contain one or more variant regions, such as CD44v6 or CD44v3-v7. It has been argued

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that CD44 may be involved in cancer metastasis through hyaluronate degradation, cellular adhesion and migration, angiogenesis, lymph node homing, and release of cytokines [12]. Moreover, an association was found between CD44 and the matrix metalloproteinase 9 (MMP-9), which is known to facilitate tumor cell invasion and metastasis [13]. Binding of hyaluronic acid (HA) of the extracellular part of the CD44 molecule to MDA-MB-231 metastatic breast cancer cells resulted in up-regulation of the growth, survival, and invasion of these cells in a study conducted by Bourguignon et al. [14]. The authors also recorded in a separate study, the expression of the CD44v3 isoform on the SP1 metastatic breast tumor cells, and reported that binding of HA to this isoform resulted in cytoskeleton-mediated tumor cell migration [15].

Several studies have investigated the CD44v3, v4, and v6 isoforms as possible prognostic markers in breast cancer, and the degree of expression of these isoforms has been examined in some histological types of the disease, but not in the IC type [7, 16–38]. .Accordingly, this study aims at examining the degree of expression of CD44v3, v4, and v6 isoforms in IC breast carcinomas, in an attempt to compare the latter with other histological types of the disease. Moreover, the possible existence of an association between these isoforms and the IC histological type is investigated.

Materials and methods

Patients and histopathological examination. The study was approved by the Human Ethics Committee of the Faculty of Medicine, Health Science Center, Kuwait University, and it conformed to the provisions of the Declaration of Helsinki.

The clinical and pathology reports and the H&E slides of breast carcinoma cases seen between 1996 and 2006 at two major public hospitals in Kuwait, namely Al-Amiri and Al-Farwaniya hospitals, were reviewed. Cases were selected where the patients' records showed that they did not receive hormonal or any other type of therapy prior to or at the time the tumor was excised. The cases were also restricted to those which were grade 1 and stage IIA (T2 N0 M0), and which had pure rather than mixed typical histological type. These selection and restriction criteria in our study protocol were mainly to control for some factors that could directly or indirectly affect the degree of expression by the tumours of the CD44v3, v4, and v6 isoforms, such as tumor grade, stage, size, hormones, and mixed histological types. The total number of cases which matched these selection and restriction criteria, and which had enough tumor tissue remaining in the paraffin blocks was 106. These included 75 invasive ductal (NOS), six invasive cribriform, six papillary, three mucinous, and 16 invasive lobular. We could not find pure tubular cases. The histological classification of the tumors as well as tumor grading and staging were determined based on the World Health Organization (WHO) guidelines and on the Elston and Ellis Method [39–40].

Immunohistochemical staining against the CD44v3, v4, and v6 isoforms. Immunohistochemical staining was performed using a highly-sensitive immunostaining kit (NovoLink Max Polymer DetSys RE7280-K, Novocastra, Newcastle upon Tyne, UK), and the steps followed were according to the manufacturer's guidelines. Sections were cut at 4 μm thickness, mounted onto silane-coated slides (S21.1910.110, Novocastra), and left to dry overnight at 37°C. They were then deparaffinized, re-hydrated, and underwent antigen retrieval (Epitope Retrieval pH6, RE7115, Novocastra) by microwaving (Daewoo KOR-161G, 1000W, 2450 MHz, 10 power levels, Seoul, South Korea) for 30 minutes. After cooling down to room temperature, the sections were incubated for 15 minutes with 3% hydrogen peroxide (Peroxidase Block RE7157, Novocastra) to block any endogenous peroxidase activity, washed with TBS, and incubated with goat serum (Protein Block RE7158, Novocastra) for 20 minutes. They were then washed with TBS, and incubated with 200 μl of primary antibody for 1 hour at 37°C in a humidified rotator. The manufacturer's recommended dilution of the primary antibody against CD44v3 (Clone VFF-327v3, Novocastra) and CD44v4 (Clone VFF-11, Novocastra) was 1:100, while that against CD44v6 (Clone VFF-7, Novocastra) was 1:50. The sections were then washed with TBS, and incubated with the post primary antibody (RE7159, Novocastra) for 30 minutes. This was followed by washing with TBS and incubation with the tertiary antibody (NovoLink Polymer RE7161, Novocastra) for 30 minutes. Finally, the sections were washed with TBS, and incubated for 10 minutes with the DAB chromogen (RE7162, Novocastra) followed by hematoxylin counterstain. Positive and negative control slides were used in each staining run. The positive controls were normal lymphocytes and samples of breast carcinomas known to be positive for CD44v3, v4, and v6. The negative controls included samples of breast carcinomas known to be negative for CD44v3, v4, and v6, and sections taken from the same tissue block but incubated with the antibody diluent instead of the primary antibody.

Scoring of immunohistochemical staining against the CD44v3, v4, and v6 isoforms. The literature has cited several studies in which scoring of immunostaining of various CD44 isoforms in human breast cancer tissues was performed. We followed the methodology recently published by Auvinen et al. [36], which reproduced that of Schumacher et al [17]. The proportion of CD44v3, v4, and v6-positively-stained tumor cells was estimated and classified as negative (0% of cells positive), weak (<10% of cells positive), moderate (10-50% of cells positive), and extensive (>50% of cells positive). Such classification was then recorded as 0-3 for practical and statistical purposes as follows: 0 (0% of cells positive), 1 (<10% of cells positive), 2 (10-50% of cells positive), and 3 (>50% of cells positive).

Statistical analysis. We performed all statistical analyses using STATA (SE 8.2, StataCorp, College Station, TX, USA). Five percent was used as the threshold for statistical signifi-

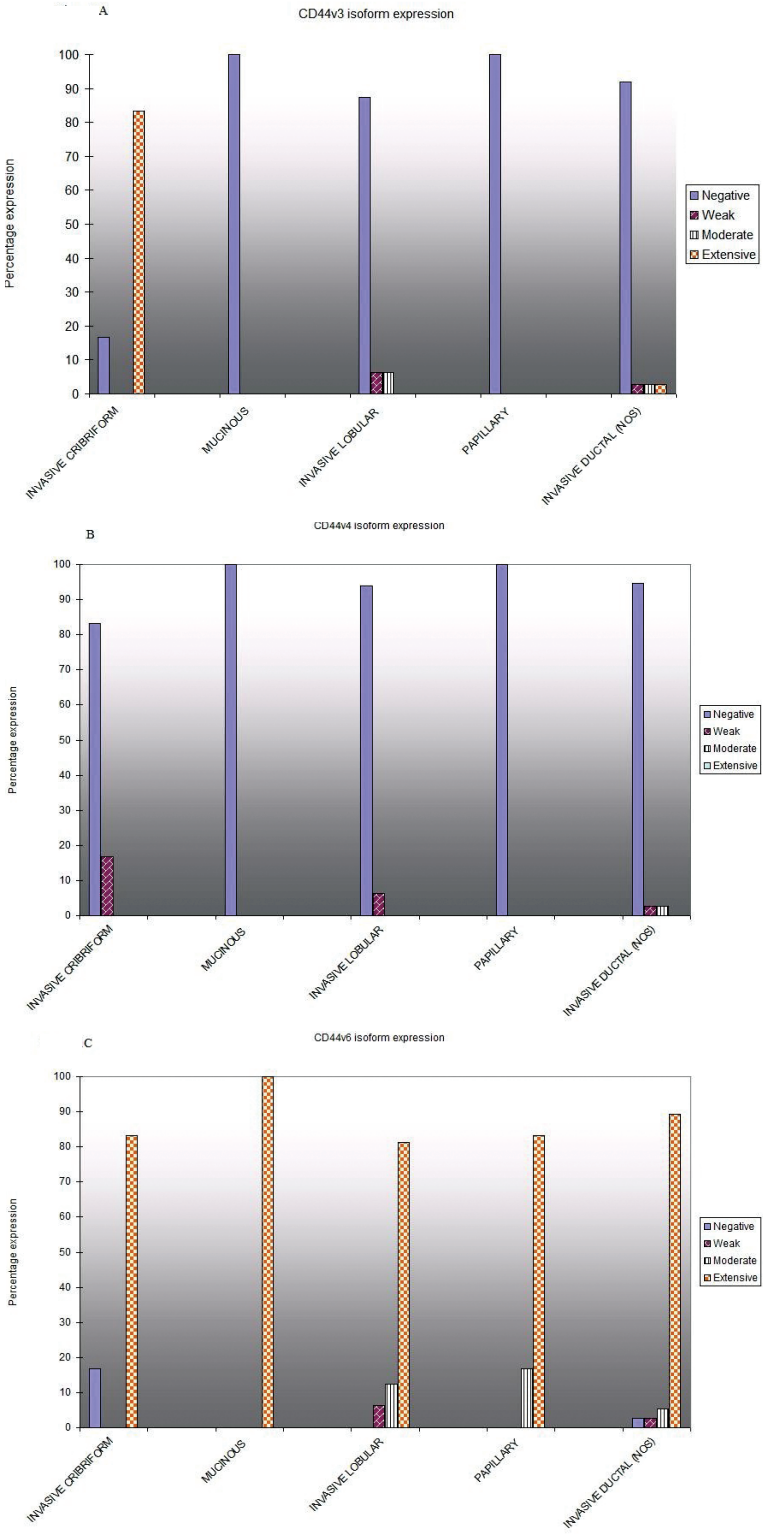

Fig. 1. Percentage expression of the CD44 isoforms in the various histological types examined.
A. Percentage expression of the CD44v3 isoform.
B. Percentage expression of the CD44v4 isoform.
C. Percentage expression of the CD44v6 isoform
Negative = 0% of cells positive; Weak = <10% of cells positive; Moderate = 10-50% of cells positive; Extensive = >50% of cells positive.

cance. The Fisher's exact test was used to assess any possible association between the degree of expression of CD44v3, v4, and v6 isoforms and histological type. The Kruskal-Wallis analysis of variance test was used to compare the actual median values of CD44v3, v4, and v6 isoform expression among the various histological types, while the Mann-Whitney test was used to compare such values between two histological types at a time. The Spearman's rank correlation coefficient, r_s, was used to determine any possible correlation among the v3, v4, and v6 isoforms.

Results

CD44v3 isoform (figs. 1A, 2). A significant association was found between the degree of expression of the CD44v3 isoform and the histological type variable. The majority (83.3%) of the IC tumours showed extensive (> 50% of the cells positive) expression of this isoform, while negative expression (0% of the cells positive) was seen only in 16.7% of the tumors. On the other hand, negative expression of the isoform was seen in 100% of each of the mucinous and papillary types, and in 92% and 87.5% of the invasive ductal (NOS) and invasive lobular types, respectively. The comparison of the actual median values of CD44v3 isoform expression revealed a significant difference among various histological types. The median value in the IC tumours was 95 (range 0, 100) as compared to 0 in the other histological types. In particular, a significant difference was found between the IC and papillary histological types, IC and invasive lobular histological types, and IC and invasive ductal (NOS) histological types. No significant difference was found between the IC and mucinous histological types, and this could be due to the small sample size in both groups.

CD44v4 isoform (figs. 1B, 3). There was no significant association between the degree of expression of the CD44v4 isoform and histological type variable, despite the fact that negative expression of this isoform was seen in 83.3% of the IC tumours, 100% of the mucinous tumours, 93.8% of the invasive lobular tumours, 100% of the papillary tumors, and 94.7% of the invasive ductal (NOS) tumors. Similarly, no significant difference was found among the actual median values of CD44v4 isoform expression of the various histological types studied.

CD44v6 isoform (figs. 1C, 4). Similarly to the CD44v4 isoform, no significant association was found between the degree of expression of the CD44v6 isoform and different histological types variable, despite the fact that such expression was

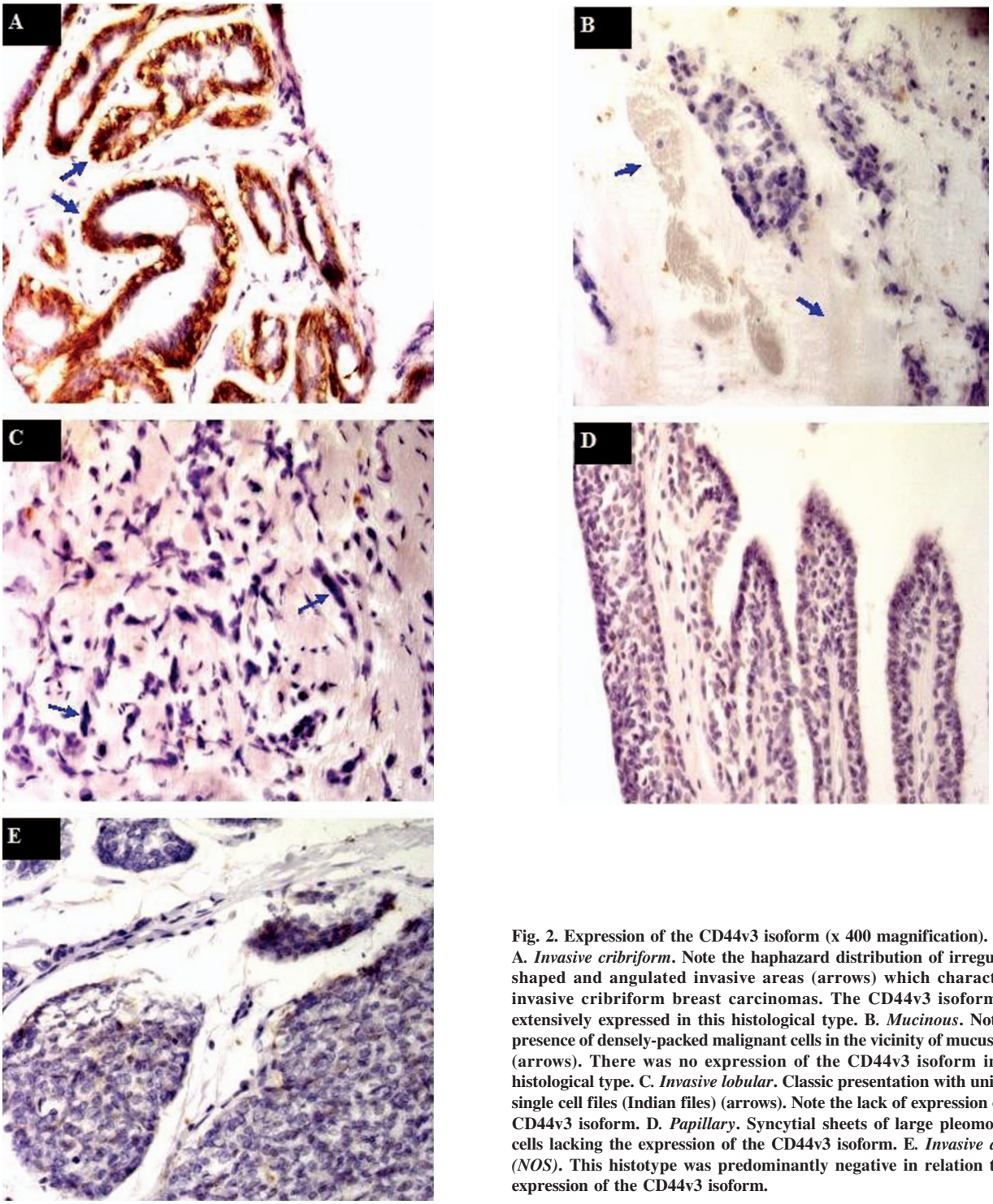

Fig. 2. Expression of the CD44v3 isoform (x 400 magnification).
A. *Invasive cribriform*. Note the haphazard distribution of irregularly-shaped and angulated invasive areas (arrows) which characterize invasive cribriform breast carcinomas. The CD44v3 isoform was extensively expressed in this histological type. B. *Mucinous*. Note the presence of densely-packed malignant cells in the vicinity of mucus lakes (arrows). There was no expression of the CD44v3 isoform in this histological type. C. *Invasive lobular*. Classic presentation with uniform, single cell files (Indian files) (arrows). Note the lack of expression of the CD44v3 isoform. D. *Papillary*. Syncytial sheets of large pleomorphic cells lacking the expression of the CD44v3 isoform. E. *Invasive ductal (NOS)*. This histotype was predominantly negative in relation to the expression of the CD44v3 isoform.

extensive (> 50% of the cells positive) in 83.3%, 100%, 81.3%, 83.3%, and 89.3% of the IC, mucinous, invasive lobular, papillary, and invasive ductal (NOS) tumors, respectively. Moreover, comparison of the actual median values of CD44v6 isoform expression revealed no significant difference among various histological types. The Spearman's rank correlation test confirmed the different trend in the expression of the isoforms v4 and v6 in our study, whereby there was a significant negative correlation between these isoforms (r_s = - 0.201).

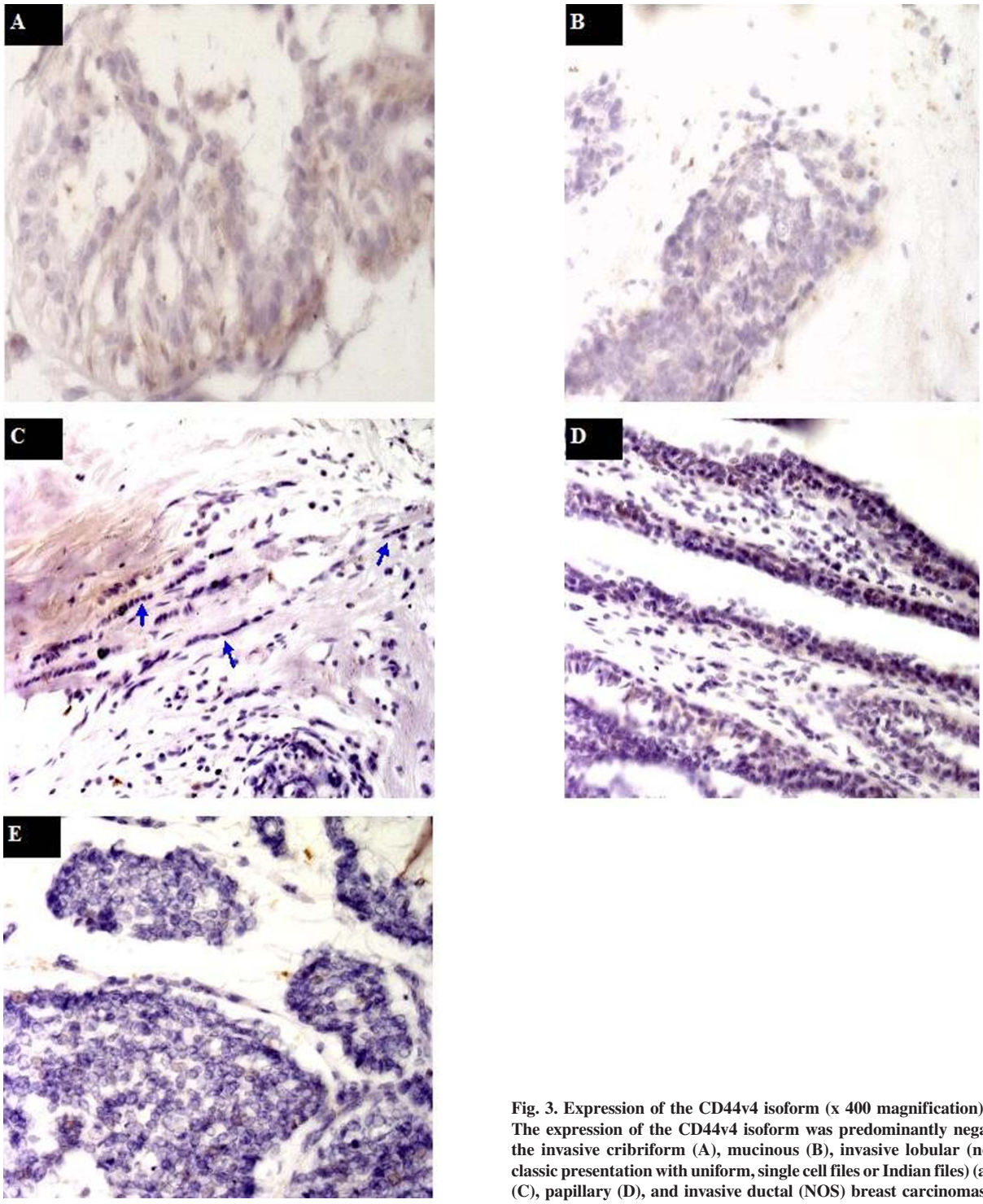

Fig. 3. Expression of the CD44v4 isoform (x 400 magnification). The expression of the CD44v4 isoform was predominantly negative in the invasive cribriform (A), mucinous (B), invasive lobular (note the classic presentation with uniform, single cell files or Indian files) (arrows) (C), papillary (D), and invasive ductal (NOS) breast carcinomas (E).

Discussion

Some histological types of breast carcinoma reflect a more favourable prognosis than others [40]. The 10-year overall survival rate in patients with IC carcinomas ranges between 90% and 100%, and the long-term prognosis for patients with breast carcinoma of the tubular histological type is similar to age-matched women without breast cancer [1–3, 41]. Patients with mucinous breast carcinomas have a 10-year survival rate ranging between 80% and 100% [42–44]. Some studies have

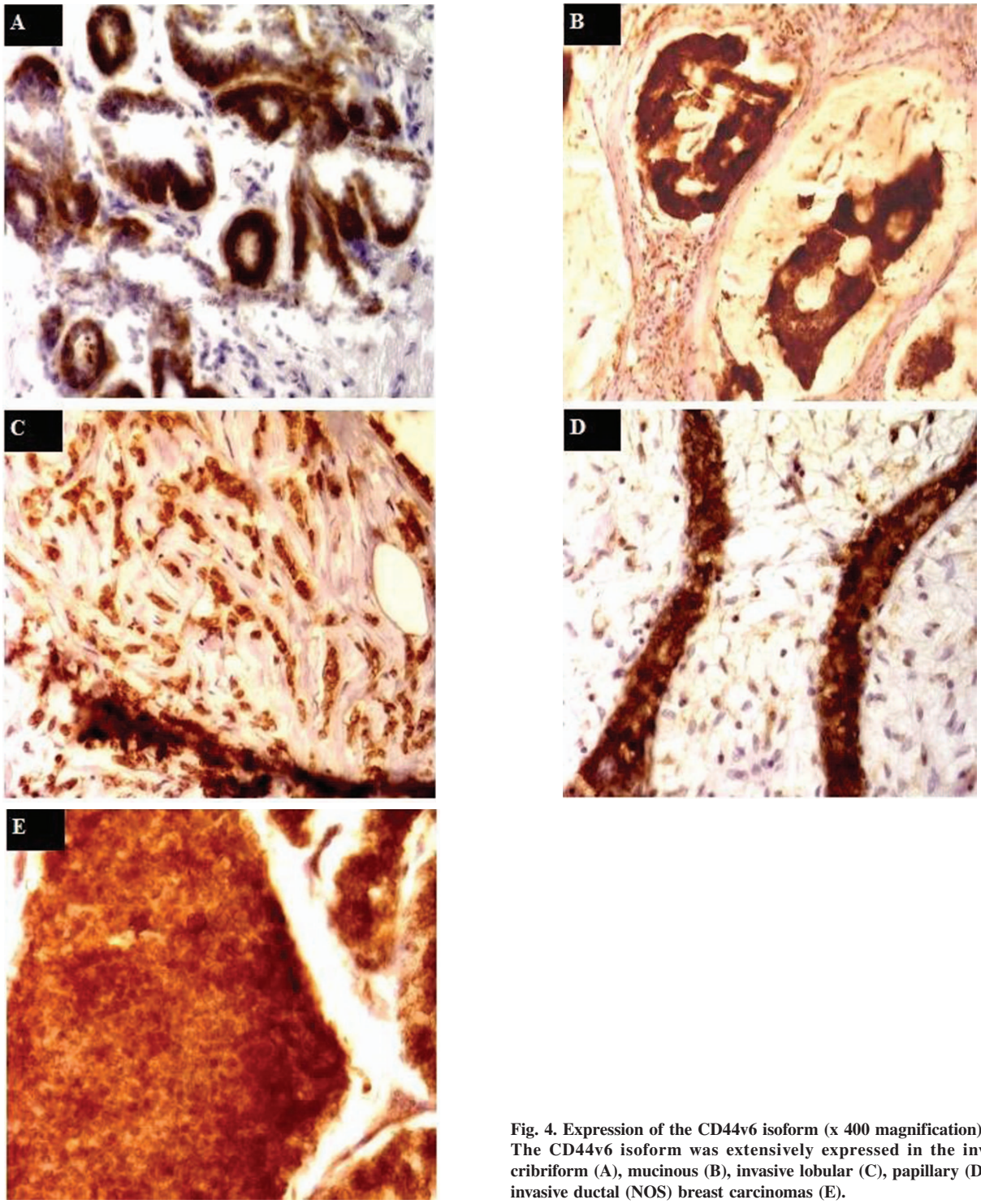

Fig. 4. Expression of the CD44v6 isoform (x 400 magnification). The CD44v6 isoform was extensively expressed in the invasive cribriform (A), mucinous (B), invasive lobular (C), papillary (D), and invasive ductal (NOS) breast carcinomas (E).

reported a better disease outcome for invasive lobular breast carcinomas as compared to invasive ductal NOS, while others have reported a worse prognosis [45–47]. Patients with medullary breast carcinomas have an overall 10-year survival rate of 50%, not exceeding 90% even in the early stages disease [3, 48–52]. Moreover, the outcome of patients with medullary carcinoma in the presence of more than three positive axillary lymph nodes has been reported to be poor [3, 48–53].

Various prognostic markers have been examined in different histological types of breast cancer, and some of these

markers were found to be associated with some of these types [54-56]. In a recent study conducted by Jalava et al. [56], the expression of oestrogen receptors was found to be significantly higher in lobular than in ductal tumors. Other studies have reported that the mucinous type was associated with an increase in the expression of oestrogen receptors, and with a decrease in the expression of HER-2 (also known as C-erb B-2 or Her-2/neu) protein [55]. Similarly, Her-2/neu was found to be inversely associated with oestrogen receptor status, based on the breast cancer histological type, in a study conducted by Coradini et al. [54] In addition to hormone receptors and Her-2/neu, the transmembrane molecule CD44 has emerged as another possible prognostic marker in breast cancer. In a recent study conducted by Sheridan et al. [57], breast cancer cells expressing CD44 were found to have both high levels of proinvasive genes and invasive properties. Similarly to the studies trying to correlate the degree of expression of some prognostic markers such as hormone receptors and Her-2/neu with the histological type in breast cancer, a number of studies have attempted to establish a possible correlation between the degree of expression of various CD44 isoforms and some breast cancer histological types, but not the IC type [7, 17, 19, 21, 25, 28, 29, 31, 33, 35-37]. Bassarova et al. [37] examined the expression of the CD44v3, v4, and v6 isoforms in breast carcinomas having the following histological types: invasive ductal (NOS), mucinous, lobular, and medullary. The authors found that the expression of CD44v3 isoform was negative in 50% of the invasive ductal carcinomas (NOS) studied, in 100% of the mucinous and medullary types, and in 60% of the lobular type. The expression of CD44v4 isoform was negative in 100% of the invasive ductal (NOS), mucinous, and medullary carcinomas, and in 80% of the lobular histological type. In contrast, the expression of CD44v6 isoform was positive in 100% of the above various histological types [37].

Our results showed that IC breast carcinomas seem to have extensive expression of the CD44v3 isoform, which was weak or lacking in the mucinous, invasive lobular, papillary, and invasive ductal (NOS) histological types. Moreover, there was a significant association with the degree of expression of this isoform. In a study conducted by Auvinen et al. [36] concerning the relation of the expression of the CD44v3 isoform, the authors reported no significant difference among invasive ductal (NOS), mucinous, lobular, medullary, ductal carcinoma *in situ*, and lobular carcinoma *in situ* tumours. Berner et al. [29] reported findings close to that of Bassarova et al. [37], whereby the percentage expression of the CD44v3 isoform was 59% in lobular breast carcinomas in the former study and 40% in the latter. Similarly, the percentage expression of this isoform in lobular carcinomas was 52% in a study conducted by Kaufmann et al. [7]. Since patients with IC breast cancer often have excellent prognosis, one could argue that the extensive expression of the CD44v3 isoform in the IC tumos in our study may, therefore, reflect good rather than bad prognosis. This possible argument is supported by the study conducted by Bassarova et al. [37], where strong expression of the CD44v3 isoform was not associated with metastases. It is also supported by Auvinen et al. [36], who reported lack of an association between this isoform and tumor grade. Other studies, however, have shown that the expression of the v3 isoform correlates with poor overall survival, increased tumor grade, and presence of metastases to lymph nodes [7, 16, 25, 26, 34, 58].

As far as the degree of expression of the CD44v4 isoform is concerned, our study revealed that the IC histological type did not significantly differ from the other histological types which we examined. The IC tumors were mostly negative for the v4 isoform, and so were the mucinous, invasive lobular, papillary, and invasive ductal (NOS) tumors. However, an association between lack of expression and histotype did not reach significance. Our results confirm those published by Bassarova et al. [37], whereby the mucinous, invasive lobular, papillary, and invasive ductal (NOS) tumors were mostly negative for this isoform. Bankfalvi et al. [25] also reported no significant difference between the lobular and ductal breast carcinomas in relation to the expression of the v4 isoform. Based on our results and those reported by Bassarova and Bankfalvi, the CD44v4 isoform may not be a prognostic marker which could be used to distinguish the biological behaviour of various histological types in breast cancer. In fact, Regidor et al. [59] reported no significant correlation between the expression of the v4 isoform and lymph node involvement or tumour grade. Similarly, Thanakit et al. [13] reported no significant difference in the expression of this isoform in high grade node positive and node negative tumors. In contrast, Sinn et al. [16] reported that the degree of expression of the v4 isoform correlated with increased tumor grade.

Our results showed that the degree of expression of the CD44v6 isoform in the IC histological type was not significantly different than that of other histological types that we examined. Such expression was extensive in most (81.3% to 100%) of the IC and other histological types. Nevertheless, no significant association was found between v6 isoform expression and histotype. Extensive expression of the v6 isoform was seen in 100% of mucinous tumors, 60% of lobular tumors, and 50% of invasive ductal (NOS) and papillary tumors in a study conducted by Bassarova et al. [37]. Another study revealed that extensive expression of this isoform was seen in 57% of mucinous tumours, 20% of lobular tumors, 38.1% of invasive ductal (NOS) tumors, and 100% of papillary tumors [28]. Still, other studies demonstrated no significant difference in the expression of the v6 isoform among the various breast cancer histological types, as well as no significant association between such expression and histotype [17, 19, 21, 25, 33, 35, 36]. Similarly to the v3 isoform, our results could possibly imply that expression of the v6 isoform may not reflect bad prognosis, since it was extensively expressed in the IC histological type, which is known to have excellent prognosis. The fact that in our study similar extensive expression of the v6 isoform seen in the IC tumors was also observed in

other histological types, which are known to have worse prognosis than the IC histotype, could not be explained at this stage of the study. Nevertheless, our results confirm those reported by other studies in which the expression of the v6 isoform did not reflect a bad prognosis, since it was found not to correlate with tumor grade, stage, size, lymph node involvement, and clinical outcome [21, 35, 36, 59–61], to be associated with less aggressive tumors and with prolonged disease-free survival [6], and to be expressed to an equal degree in both node-positive and node-negative non-palpable T1a and T1b invasive ductal (NOS) carcinomas [62]. Other studies, however, reported that increased v6 isoform expression in breast cancer was associated with metastasis [20, 22, 24, 27, 32], and that such expression correlated with poor overall survival and tumor grade [7, 16, 18, 25, 30]. In a recent study conducted by Ma et al. [38], the authors reported a sequential increase in the expression of the v6 isoform in women with metastases in the axillary lymph nodes, tumor size above 2 cm, advanced pTNM stage, and a survival period of less than five years. Kopp et al. [30] reported that the degree of expression of the v6 isoform was significantly associated with the number of metastasized organs, presence of hepatic metastases, and poor response to chemo- and hormonal therapy. In our study, we observed a significant negative correlation between the v4 and v6 isoforms, which confirms our results whereby there was negative expression of the former in the IC as well as other histological types as compared to extensive expression of the latter.

In conclusion, our results have demonstrated, for the first time in the literature, that IC tumors significantly differ from other histological types of breast cancer in relation to the degree of expression of the CD44v3 isoform, but not of the v4 or v6 ones. Having controlled, in our study, for confounding variables (tumour grade, stage, size, and lymph node involvement) which could affect the degree of expression of these isoforms by analysing tumors which were all grade 1 and stage IIA (T2 N0 M0), our results may therefore reflect the actual biological behaviour of the IC histological type. We acknowledge the fact that we have a small number of cases in some of the histological groups which we studied. This is due to two main reasons. First, it is known that the occurrence of some histological types of breast cancer is rarer than other histological types. Second, we faced tremendous difficulties trying to recruit specimens which could match our strict selection criteria. The clinical translation of our observations could be in drawing the attention of the oncologists in relation to focusing more on the CD44v3 isoform as compared to other metastatic markers. This is based on the following four logical links, whether the expression of this isoform is predominant in the IC tumours, metastasis in the IC tumours do not exceed 14.3%, the 10-year overall survival of the patients with IC tumours is between 90% and 100%, and if other more aggressive histological types such as mucinous, papillary, invasive lobular, and invasive ductal (NOS) rarely express this isoform.

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