Expression of CD44 variants in osteosarcoma

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Abstract The standard form of CD44 (CD44H) is a transmembranous glycoprotein, widely distributed on a variety of human lymphoid cells, epithelial cells and tumours. CD44 has many variant forms, which are generated by alternative splicing. In recent years, CD44 has been reported to be related to the degree of tumour differentiation, tumour cell invasion, and metastasis. We investigated 44 tumour specimens in 39 patients with osteosarcoma immunochemically to analyse the expression of CD44 standard (CD44H) and variant exon-encoded gene products (CD44v3, v4, v5, v6, v7, v9, and v10). Furthermore, the relationship between CD44 expression and the clinical outcome of patients with osteosarcoma was analysed. Membrane accentuation and exclusive cytoplasmic reactivity were analysed as separate staining patterns. Tumour cells and some multinucleated giant cells were markedly stained. CD44H, v3, v4, v5, v6, v7, v9, and v10 were expressed in 85%, 49%, 54%, 59%, 46%, 5%, 28%, and 10% of the specimens respectively. The cumulative 5-year metastasis-free survival was 58% in CD44v6-negative cases and 24% in CD44v6-positive cases (P = 0.046). However, the cumulative 5-year metastasis-free survival was not significantly different between cases positive and negative for other variants of CD44. Multivariate analysis (Cox proportional-hazard model) with CD44v6 expression (positive or negative), chemotherapy (intensive or non-intensive), tumour site (proximal or distal), and age (at least 30 years or less than 30 years) showed that expression of CD44v6 and chemotherapy were important prognostic factors in patients with osteosarcoma. Overexpression of CD44 isoforms containing variant v6 is correlated with poor prognosis in patients with osteosarcoma.

Key words Osteosarcoma · CD44 · Immunohistochemistry · Prognosis

Introduction

CD44 is the cell-surface receptor for cell adhesion, proliferation and metastatic tumour growth and it has also been associated with the degree of cellular differentiation (Jalkanen et al. 1986; Isacke et al. 1986; Lucas et al. 1989; Picker et al. 1989; Miyasaki 1995). CD44 exists on the surface of both haematopoietic cells and non-haematopoietic cells including subsets of leucocytes, erythrocytes, and epithelia (Belitsos et al. 1990; Güntchert 1993; Jalkanen et al. 1991; Aruffo et al. 1990). CD44 is also present in mesenchymal cells such as fibroblasts, smooth muscle cells, and glial cells of the central nervous system. The commonest type of CD44 is the “standard” or haematopoietic variant (CD44s or CD44H) (molecular-mass range, 80–90 kDa), which is expressed by cells of mesodermal origin (haematopoietic, fibroblastic, and glial cells) and by some carcinoma cell lines (Picker et al. 1989). CD44 has many variant forms, which are generated by alternative splicing of at least 10 exons named v1–v10 (Arch et al. 1992). Recent genomic cloning has revealed the CD44 gene to contain a total of 19 exons, 12 of which can be alternatively spliced resulting in numerous CD44 variants at the protein level (Screaton et al. 1992).
poor prognosis was reported (Dall et al. 1995; Joensuu et al. 1993a; Kainz et al. 1995; Mayer et al. 1993; Wielenga et al. 1993). In the work described here we examined the expression and localization of CD44 variant exons in osteosarcoma tissues. The relationship between CD44 expression and the clinical outcome of osteosarcoma patients was also analysed.

**Materials and methods**

**Patients**

For each patient with osteosarcoma treated between 1980 and 1996, the medical records, histological sections, and radiographs were reviewed. All osteosarcomas were classified as conventional high-grade tumours. Intensive pre- and postoperative chemotherapy was performed. The subjects were 39 patients for whom tumour (paraffin-embedded) tissues and information from follow-up examinations were available and who did not have visible metastasis on chest radiography, computed tomography (CT), or bone scans at diagnosis. Only 1 patient, treated in the early 1980s and still alive, was not examined by chest CT.

Of the 39 patients, 22 were male and 17 were female. The mean age at initial surgery was 19 years (range, 8–55); patients at least 30 years of age were defined as the aged group and those below 30 years as the young group. Twenty-nine tumours were in the femur, 9 in the tibia, and 1 in the fibula; the 29 tumours of the distal to the knee joint were regarded as distal tumours. Thirty-two patients had osteoblastic, 4 had chondroblastic, and 3 had fibroblastic osteosarcoma. All patients had stage IIB tumours according to the criteria of Enneking et al. (1986). One patient had a local relapse before development of metastasis. The follow-up period ranged from 24 months to 168 months with an average period of 70.9 months. In addition, 1 specimen of the local relapse and 4 specimens of metastases were examined.

**Chemotherapy**

All patients received systematic chemotherapy. Between 1980 and 1989, the chemotherapy was composed of doxorubicin and high-dose methotrexate. After 1990, patients received anticancer drugs comprising doxorubicin, high-dose methotrexate and cisplatin. These protocols were previously reported (Uchida et al. 1999). Twenty-one patients received intensive chemotherapy and 18 did not. Patients treated after 1990 were mainly regarded as having received intensive chemotherapy. No patient received radiotherapy to the primary lesion.

**Surgery**

Eighteen patients treated before 1990 underwent amputation; 1 had a local relapse before developing metastasis. In 21 patients treated after 1990, 7 underwent amputation, 6 arthrodesis, 4 rotationplasty, 2 endoprosthesis and 2 implantation of the irradiated bone.

**Immunohistochemistry**

A total of 44 specimens including 39 samples from 39 primary lesions and 5 samples from the 5 relapsed lesions were immunohistochemically analysed for the expression of CD44H and its splicing variants. The tumour was cut at the largest plane and divided between several slides. Two or three slides with the most aggressive and active tumour cells were selected for each case in this examination. Antigen retrieval was performed by autoclaving the sections in citrate buffer at a pH of 6.0 for 15 min at 121°C.

Monoclonal antibodies against CD44H, v3, v4, v5, v6, v7, v9, and v10 were used as primary antibodies (CD44H clone 2C5, R&D Systems; CD44v3 clone VFF-327v3, CD44v4 clone VFF-11 and CD44v5 clone VFF-8, Bender MedSystems; CD44v6 clone 2F10, R&D Systems; CD44v7 clone VFF-9, Bender MedSystems; CD44v9 clone 441V; CD44v10 clone VFF-14, Bender MedSystems).

After blocking with diluted normal blocking serum, the sections were incubated for 1 h with a 1:200 dilution of primary antibodies at 37°C. After rinsing in phosphate-buffered saline (PBS), sections were sequentially incubated for 30 min with diluted biotinylated secondary antibody solution. Slides were washed for 5 min in PBS and sections were incubated for 30 min with ABC Regent [Vectastain Elite ABC Kit (mouse IgG); Vector Laboratories, Burlingame, Calif, USA]. The antigen-antibody binding was revealed by immersing sections in diaminobenzidine hydrochloride with 0.6% hydrogen peroxide as a substrate [Histofine SAB-PO (M) Kit; Nichirei Corporation, Tokyo, Japan] for 5 min. The sections were finally counterstained with Mayer’s haematoxylin to make the nuclei visible, then rinsed in tap water for 10 min, dehydrated in a graded alcohol series (70%–absolute methanol) and through xylene, and covered.

**Positive and negative controls**

The positive control slide was prepared from colon cancer or breast cancer known to contain the antigen (Joensuu et al. 1993a; Wielenga et al. 1993; Kaufmann et al. 1995; Tanabe et al. 1993). In the positive control tissue, all monoclonal antibodies were applied under the same condition as the osteosarcoma samples. The negative control slide was prepared from the same tissue block as the specimen. Instead of the primary antibody, we used non-immune mouse monoclonals of the same isotype as the specific antibodies.

**Quantification**

Immunoreactivity was evaluated by light microscopy without knowledge of the treatment outcome or other data of the patients. A tumour was considered positive if staining was confined to the cell membrane or the cytoplasm in the absence of significant background staining, and if staining occurred in more than 10% of the tumour cells. CD44 expression was scored visually, using semi-quantitative gradings, according to the following criteria: – no staining, + staining in 10%–25% of tumour cells, ++ staining in 25%–75% of tumour cells, +++ staining in more than 75% of tumour cells. Moreover, in each of the patterns, the staining of the cell membrane and of the cytoplasm were recorded separately. In cases when both the cytoplasmic and membranous staining were positive, the more predominantly stained site was described. In some tumours, heterogeneity in CD44 reactivity was noted between different areas of the same tumour sample. In such cases, the most strongly positive area with CD44 was usually analysed. A consensus was reached by three of the authors in all cases.

**Statistics**

Statistical significance of the difference between groups was evaluated by the $\chi^2$-test and $P < 0.05$ was considered significant. The cumulative probability of metastasis-free survival was calculated by the Kaplan-Meier method. Univariate log-rank analysis was used to compare curves. The multivariate regression test, according to the proportional-hazard model of Cox, was performed to identify the factors influencing the prognosis of patients with osteosarcoma.