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Immunolocalization of heparin-binding EGF-like growth factor (HB-EGF) as a possible immunotarget in diagnosis of some soft tissue sarcomas

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ABSTRACT

Heparin-binding EGF-like growth factor (HB-EGF), a member of the family of epidermal growth factors (EGFs), is involved in several biological processes and tumor formation. Several lines of evidence show that HB-EGF plays a key role in the acquisition of malignant phenotype. Studies show that HB-EGF expression is essential in oncogenesis of cancer-derived cell lines. HB-EGF is a promising target for cancer therapy. The aim of this study was to find new insights on the biological features of the soft tissue sarcomas, in order to consider the possibility to use HB-EGF as an immuno-target in histotype characterization and to facilitate therapeutic intervention. In our study we did HB-EGF-immunostaining on tissue samples collected from 43 human soft tissue sarcomas. We analyzed HB-EGF immunoexpression in some types of tumors such as clear cell sarcomas, leiomyosarcomas, phyllodes sarcomas, chondrosarcomas and liposarcomas. In relation to the different histotypes, we detected different immunostaining localization. From our results it was evident that pleomorphic cells, a signal of tumor progression, were HB-EGF immunostained, and this was accompanied by an extracellular matrix immunostaining. Moreover statistical analysis showed a correlation between HB-EGF immunostaining and the different types of analyzed soft tissue sarcomas. In conclusion, in some types of soft tissue sarcoma HB-EGF could be considered a useful diagnostic marker for their characterization.

Introduction

HB-EGF is a growth factor belonging to the family of EGFs. It is synthesized as a transmembrane precursor (proHB-EGF) by macrophages (Higashiyama et al., 1991; Massague and Pandiella, 1993). A protease cleaves proHB-EGF to produce the soluble form of HB-EGF (sHB-EGF) through the "ectodomain shedding mechanism" (Goishi et al., 1995). sHB-EGF is a potent mitogen and chemoattractant for different cell types (Raab and Klagsbrun, 1997). Studies in mice expressing proHB-EGF show that the main functions of HB-EGF are due to the soluble form (Yamazaki et al., 2003). ProHB-EGF can transmit positive or negative biological signals to neighboring cells by a juxtacrine mechanism and studies show that an important role for its function is played by the cytoplasmic domain (Higashiyama et al., 1995; Iwamoto et al., 1999). The latter is phosphorylated by external stimuli, and the phosphorylation site plays a role in the mechanism of oncogenesis (Wang et al., 2006). HB-EGF is involved in different physiological and pathological processes (Miyagawa et al., 1995; Raab and Klagsbrun, 1997; Paria et al., 1999; Shirakata et al., 2005; Mine et al., 2005; Kimura et al., 2005) and its expression has been demonstrated in different tissues, such as skin, lung, heart, brain and skeletal muscle (Abraham et al., 1993; Castrogiovanni et al., 2011). As already referred to, ectodomain shedding from proHB-EGF is necessary for sHB-EGF activity (Yamazaki et al., 2003). In addition, stimuli inducing proHB-EGF shedding ensure activation of EGF receptors (EGFRs), a potential cause of oncogenesis (Miyamoto et al., 2006; Yotsumoto et al., 2008). Several studies have shown that HB-EGF is involved in proliferation of tumor cells (Xu et al., 1998; Fu et al., 1999; Harding et al., 1999; Keates et al., 2001; Wallasch et al., 2002; Means et al., 2003; Busiello et al., 2004; Miyamoto et al., 2004; Tanaka et al., 2005; Yagi et al., 2005; Thogersen et al., 2001; Kobrin et al., 1994). Furthermore, *in vitro* experiments show that an anti-HB-EGF blocking antibody reduces proliferation by 30–40% in human glioblastoma cells (Mishima et al., 1998). Inhibition of HB-EGF expression in myeloma cells treated with CRM197, a non-toxic

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mutant of diphtheria toxin, results in suppression of cell proliferation and induction of cell apoptosis (Wang et al., 2002; Ichise et al., 2010). There are many lines of evidence that sHB-EGF plays a strong role in cell motility, but not in cell proliferation, and it also plays fundamental roles in cell migration and invasion (Higashiyama et al., 1993; Shirakata et al., 2005; Mine et al., 2005). In cancer, it contributes to the tumor aggressiveness through interactions with several molecules. Consequently HB-EGF could be one of the target candidates for metastasis (Tarbe et al., 2002). *In situ* hybridization and immunohistochemistry show that HB-EGF expression is elevated in human hepatocellular carcinoma biopsies (Ito et al., 2001); in colon and pancreatic cancers, expression of HB-EGF is associated with the early stage (Uchida et al., 1971); in ovarian cancer, HB-EGF immunostaining is evident in interstitial tissues, but not in cancer tissues, indicating that most of the HB-EGF is shed from the cell membrane in cancer cells and deposited in interstitial tissues (Tanaka et al., 2005; Miyamoto et al., 2006; Ichise et al., 2010). High expression of HB-EGF has also been detected in some soft tissue sarcomas and it is significantly correlated to a shorter overall survival and therefore to an unfavorable prognosis (Hoffmann et al., 2009). It is well known that the expression or activation of EGFR and ErbB2 receptor are impaired in many epithelial tumors, and that these receptors have an important role in oncogenesis. HB-EGF is more expressed, compared to other EGFR ligands, in different types of cancer, thus the use of HB-EGF as a target for tumors could be more effective than the use of EGFR molecules (Miyamoto et al., 2006). Furthermore, studies suggest that HB-EGF is a major ligand among the EGFR ligands in some kinds of cancer (Thogersen et al., 2001; Miyamoto et al., 2004; Tanaka et al., 2005), therefore HB-EGF could be a promising target for diagnosis (Miyamoto et al., 2006).

Soft tissue sarcomas represent a heterogeneous group of mesenchymal and rare, malignant tumors. Correct clinical management of these tumors should require information from clinical investigations, imaging, histopathology, and cytogenetic and molecular genetic analyses. Although morphological findings, immunophenotyping and karyotyping are very helpful in the diagnosis of some soft tissue sarcoma subtypes, other subtypes, such as malignant fibrous histiocytoma, leiomyosarcoma and dedifferentiated/pleomorphic liposarcoma, are more difficult to diagnose (Beck et al., 2010). Pre-operative chemotherapy may be considered for those patients with large high grade tumors that are considered borderline resectable (Salgado and van Marck, 2006). If the tumor is chemosensitive and adjacent to critical organs then chemotherapy may render the tumor suitable for conservative surgery, otherwise more radical surgery may be necessary (Karavasilis et al., 2008). There is a wide variation in chemosensitivity between different histological subtypes, in fact there are several lines of evidence for the differential response to chemotherapy according to histological subtype. For example, synovial sarcoma, leiomyosarcoma and myxoid liposarcoma are recognized as having higher response rates to chemotherapy, on the contrary alveolar soft part sarcoma, extraskeletal myxoid chondrosarcoma and solitary fibrous tumor are generally insensitive to chemotherapy and there are only occasional reports of responses in clear cell sarcoma (Leu et al., 2004; Maki, 2007; Demetri et al., 2009). Decisions on the kind of chemotherapy and choice of treatment should be based on histology and toxicity profile.

Soft tissue sarcomas are difficult to characterize because of the undifferentiated nature of this kind of tumor, and it is well known that the use of immunohistochemistry is useful for determining a particular histological type. The aim of this study was to find new insights on the biological features of the soft tissue sarcomas, in order to consider the possibility of using HB-EGF as an immunotarget in histotype characterization and to facilitate the therapeutic intervention.

Materials and methods

Patients and clinical samples

Tissue samples were collected from 43 human soft tissue sarcomas for paraffin embedded material and slides. Patients affected by soft tissue tumors between 2006 and 2011 were selected from our files. Data were analyzed anonymously. Informed consent was obtained from each patient; the research was approved by the Local Medical Ethical Committee (University of Catania) and conformed to the ethical guidelines of the Declaration of Helsinki. In the present study, we evaluated 43 cases of soft tissue tumors with clinical data, divided into five types of tumor (10 clear cell sarcomas, 15 leiomyosarcomas, 4 phyllodes sarcomas, 6 chondrosarcomas and 8 liposarcomas) for immunohistochemical analysis. Soft tissue sarcomas from histopathological archive of Policlinic of Catania were reviewed, classified according to World Health Organization 2002 criteria and graded according to the French Federation of Cancer Centers Sarcoma Group system (Bhargava et al., 2005; Yaziji and Taylor, 2007).

Wide resection margins of neoplastic masses were made, obtaining, in most of cases, peritumoral areas also submitted to histological and immunohistochemical analysis. Clinical information was available and is summarized in Table 1.

Histology

Samples were fixed in 10% buffered-formalin, following overnight wash, specimens were treated as previously described (Musumeci et al., 2012a). Sections 4–5 μm thick were obtained according to routine procedures, mounted on silane-coated slides and stored at room temperature. Slides were dewaxed in xylene, hydrated using graded ethanol, and stained for routine histological evaluation by hematoxylin and eosin (H&E) staining. The sections were examined with an Axioplan Zeiss light microscope (Carl Zeiss, Oberkochen, Germany) and photographed with a digital camera (Canon, Tokyo, Japan).

Immunohistochemistry (IHC)

Specimens following overnight washing, were dehydrated in graded ethanol, cleared in xylene and paraffin-embedded, with their anatomical orientation preserved. Sections were processed as previously described (Leonardi et al., 2012a, 2012b). Briefly they were incubated for 30 min in 0.3% H_2O_2/methanol to quench endogenous peroxidase activity then rinsed for 20 min with phosphate-buffered saline (PBS; Bio-Optica, Milan, Italy). The sections were heated (5 min × 3) in capped polypropylene slide-holders with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0; Bio-Optica, Milan, Italy), using a microwave oven (750 W) to unmask antigenic sites. The blocking step performed before application of the primary antibody with goat serum (Vector Laboratories, Burlingame, CA, USA), 1:20 work dilution in PBS-T, 1 h in a moist chamber.

Then, the sections were incubated overnight at 4 °C with Human HB-EGF affinity purified polyclonal Ab (Goat IgG) (AF-259-NA. R&D Systems, Minneapolis, MN, USA). The secondary antibody, biotinylated anti-mouse antibody was applied for 30 min at room temperature, followed by the avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for further 30 min at room temperature. The immunoreaction was visualized by incubating the sections for 4 min in a 0.1% 3,3′-diaminobenzidine (DAB) and 0.02% hydrogen peroxide solution (DAB substrate kit, Vector Laboratories, CA, USA). The sections were lightly counterstained with Mayer's Hematoxylin (Histolab Products AB, Goteborg, Sweden) mounted in GVA mount (Zymed Laboratories, San Francisco, CA,