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Mechanisms of local invasion in enteroendocrine tumors: Identification of novel candidate cytoskeleton-associated proteins in an experimental mouse model by a proteomic approach and validation in human tumors

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ABSTRACT

Small-intestinal neuroendocrine tumors (SI-NETs) are defined as locally invasive only after extension to the muscularis propria. To gain further insight into the molecular mechanisms, we applied a proteomic approach to an orthotopic xenograft model to identify candidate proteins evaluable in human SI-NETs. After grafting STC-1 neuroendocrine tumor cells on the caecum of nude mice, comparative proteomic studies were performed between the pre-invasive and the invasive stages, respectively 2 and 8 weeks after grafting. We identified 24 proteins displaying at least a 1.5-fold differential expression between 2 and 8 week-stages. Most were cytoskeleton-associated proteins, among which five showed decreasing expression levels (CRMP2, TCP1ε, TPM2, vimentin, desmin) and two increasing expression levels (14-3-3γ, CK8). Changes for CRMP2, TCP1ε, TPM2 and 14-3-3γ were confirmed in experimental tumors and in a series of 28 human SI-NETs. In conclusion, our results underline the relevance of proteomics to identify novel biomarkers of tissue invasion.

1. Introduction

Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) are uncommon neoplasms, retaining most of the phenotypic and functional characteristics of the normal peptidergic endocrine cells present throughout the digestive tract. One of the main clinical problems raised by GEP-NETs is their highly variable behavior, which ranges from benign to highly malignant. For pathologists, the challenges are therefore to identify overtly malignant tumors and to predict their risk of progression and dissemination (Modlin et al., 2008). In the digestive tract, it is recognized that one objective sign of malignancy is the presence of local invasion, because of its association with an increased risk of regional and distant metastasis. In adenocarcinomas, a tumor is defined as invasive as soon as the epithelial basement membrane of the surface epithelium is disrupted and the lamina propria of the mucosal layer is colonized; the risk of metastatic dissemination will then depend on the depth of invasion and on the organ involved (Rindi et al., 2010). In contrast, GEP-NETs are defined as locally invasive only when they extend to the muscularis propria: this extension is considered an objective sign of malignancy and is associated with a risk of regional and distant metastasis (Rindi et al., 2010).

Recently, the mechanisms of tumor cell invasion have been the subject of intensive studies (Friedl and Alexander, 2011). Several modalities have been described and the corresponding cellular and molecular events have been, at least in part, uncovered. They associate increased cell motility, cytoskeletal reorganization, matrix remodeling and changes in cell–cell and cell–matrix interactions.

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However, recent studies of tumor cell invasion have mainly focused on carcinomas and on their interactions with extracellular matrix. As a result, despite their importance in tumor progression and natural history, very little is known about the mechanisms of local invasion by gastro-intestinal NETs and especially on their interactions with the muscular layers of the digestive wall.

To gain insight into these mechanisms, we decided to focus on one location of GEP-NETs, the small intestine. Several strategies to uncover the mechanisms of local invasion in this tumor subtype could be discussed. Gene expression profiling may be useful to identify new molecular mechanisms, but the rarity of small-intestine NETs (SI-NETs) hampers the constitution of large, homogeneous and representative series of human tumors. Moreover, almost all SI-NETs are diagnosed at a locally invasive and often metastatic stage, making it impossible to study the early steps of tumor cell invasion. Finally, as for other tumors, the key molecular and cellular events involved in SI-NETs invasion are restricted in time and space and are therefore difficult to detect by using global molecular analyses. This likely explains why, so far, studies of gene expression profiles in SI-NETs have provided little information about the mechanisms of local invasion (Bloomston et al., 2004; Capurso et al., 2006; Couvelard et al., 2006; Duerr et al., 2008; Hansel et al., 2004; Maitra et al., 2003).

We therefore decided to explore an alternative strategy. We used an experimental animal model of orthotopic neuroendocrine tumor xenograft making it possible to explore the initial steps of the invasion of muscularis propria by tumor cells (Poncet et al., 2009). We then performed a proteomic analysis comparing tumor samples representative of the various stages of local invasion, in order to directly identify molecules which may be relevant from a biological and a clinical point of view. We then evaluated the candidate proteins suggested by the experimental study in a validation series of human SI-NETs.

2. Materials and methods

2.1. Experimental study

2.1.1. Cell culture and experimental animal model

The intestinal STC-1 cell line, a gift from Guido Rindi (Department of Pathology, Laboratory Medicine, Parma, Italy), is derived from a neuroendocrine tumor developed in the small intestine of RIP1Tag2/RIP2PyST1 transgenic mouse (Rindi et al., 1990). This cell line has been selected because of its origin in the small intestine, its locally invasive and metastatic behavior in experimental in vivo models, as shown in previous works from our laboratory (Poncet et al., 2009) and its typical neuroendocrine phenotype, even if its proliferative capacities are higher than those of the majority of human SI-NETs. STC-1 cell line was routinely cultured in DMEM supplemented with 5% fetal calf serum (FCS), 2 mmol·L^{-1} glutamine and antibiotics (100 UI·mL^{-1} penicillin plus 50 mmol·L^{-1} streptomycin).

The xenografting procedure was performed as previously described (Poncet et al., 2009), using STC-1 cell cultures between 20th and 30th passages, to avoid any modification of the cell phenotype. Female athymic nu/nu CD-1 nude mice at 4 weeks of age were obtained from Charles River Laboratories (L'Arbresle, France). Mice were housed and bred in a specific pathogen-free animal facility. The experiments were performed in accordance with the animal care guidelines of the European Union and French laws and were validated by the local Animal Ethic Evaluation Committee (CECCAPP). Animals were anesthetized prior to all surgical procedures. After surgery, animals were allowed to recover in a sterile atmosphere and were fed ad libitum with a sterile diet.

Briefly, solid tumors were first obtained through subcutaneous injection of STC-1 cell suspension in nude mice. Two weeks later, subcutaneous tumors were excised and cut into small pieces. These fragments were immediately grafted on the serosal surface of the caecal wall of nude mice. Animals were randomly allocated into two groups of eight animals each, with sacrifice at either 2 or 8 weeks; times of sacrifice have been selected on the basis of previous works from our group, which have shown that at 2 weeks, grafted tumor tissue was still restricted to the serosal layer and that, at 8 weeks, all grafted tumors have crossed the muscularis propria and invaded the submucosal layer (Poncet et al., 2009). At the end of the experiment, tumor tissue at the site of grafting was carefully excised under magnifying glasses and prepared for proteomic analysis ($n = 3$ in each group) and western blotting or histological investigations ($n = 5$ in each group); in addition, small samples of excised tumor tissue were preserved for histological control before proteomic analysis. Lymph nodes and liver were also removed for further histological analysis.

2.1.2. Proteomic analysis

2.1.2.1. Tissue sample preparation. Primary tumor samples were harvested in lysis buffer (8 M urea, 4% CHAPS and 40 mM Tris base) on ice. The lysate was centrifuged at 12000 rpm at 4 °C for 5 min to remove cell debris. The supernatant was taken as extracted proteins and the protein concentration was determined by the Bradford method with BSA as standard.

2.1.2.2. 2-D electrophoresis (2-DE). First dimension isoelectric focusing was carried out using an EtanIPGphor isoelectric focusing system, as described by the manufacturer. The protein quantity used for each analytic gel was 100 μg. For rehydration, protein was diluted to 340 μL in a buffer containing 8 M urea, 2% CHAPS, 0.5% ampholytes, 50 mM DTE, and 0.004% of bromophenol blue. Samples were loaded in immobilized pH 4–7 gradient (IPG) strips. After 1 h of rehydration, the strips were focused at a temperature of 20 °C using the IPGphor IEF apparatus (GE Healthcare, Orsay, France) using the following voltage settings: 20 V for 13 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 6 h. The gels containing the focused proteins were stored at –80 °C until the two-dimensional analysis or were used immediately.

The strips were brought to room temperature and equilibrated in two steps for 15 min each to reduce and alkylate the proteins. The equilibration buffer contained 6 M urea, 2 M thiourea, 50 mM Tris, 34.5% glycerol, 2% SDS, and 0.005% bromophenol blue with 2% DTT for the first step, and 2.5% iodoacetamide replaced DTT for the second step. Strips were then placed on top of a 12.5% polyacrylamide gel and sealed with 0.5% agarose dissolved in electrophoresis buffer. The DALT six apparatus (GE Healthcare, Orsay, France) was used for 2-DE at constant temperature of 22 °C (20 W for 30 min and 60 W for the remaining time of electrophoresis) until a total of 1000 Vh was achieved. After electrophoresis, the gels were stained by using the Silver Stain Plus Kit (Bio-Rad).

2.1.2.3. 2-D gel image analysis. The silver-stained 2-DE gels were scanned on an Epson scanner at a resolution of 300 dots per inch (dpi). Intensities of protein spots were analyzed with ImageMaster 2-D (GE Healthcare, Orsay, France). Protein spots that showed more than 1.5-fold statistically significant differential expression and had the same directional change in three separate experiments were taken as differentially expressed candidates.

2.1.2.4. Protein identification by mass spectrometry. Gel spots corresponding to differentially expressed proteins were excised. Proteins contained in each gel spot were reduced, alkylated and digested with modified trypsin (Promega) using the procedure described by Scherl et al. (Scherl et al., 2002). Peptides were deposited on to the MALDI targets, combined with matrix (CHCA in 50% CH_3CN, 0.1% TFA, with 10 mM $NH_4H_2PO_4$) and analyzed in MS and MS/MS (maximum of