Short Communication

Analysis of E-Cadherin in Diffuse-Type Gastric Cancer Using a Mutation-Specific Monoclonal Antibody

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In-frame deletions from the E-cadherin mRNA, coding for a homophilic cell adhesion molecule, are characteristic for diffuse-type gastric carcinomas. Using immunohistochemical analysis the mutant form cannot be distinguished from normal E-cadherin, making results difficult to interpret. In this study, a rat monoclonal antibody, designated E-cad delta 9–1, was generated against a peptide spanning the fusion junction region between exons 8 and 10. This new epitope is present in an E-cadherin variant that lacks exon 9 from the mRNA due to different splice-site gene mutations. Using Western blotting and immunohistochemistry of E-cadherin-transfected cells, we demonstrate that E-cad delta 9–1 specifically reacts with E-cadherin lacking exon 9 but not with the wild-type protein. No immunoreactivity was observed in 31 nontumorous and embryonal tissues analyzed. In gastric carcinoma specimens known to express mutant E-cadherin mRNA lacking exon 9, E-cad delta 9–1 targets exclusively tumor cells in routine formalin-fixed and paraffin-embedded material from biopsies, primary tumors, and lymph node metastases. In a retrospective series of 172 diffuse-type gastric carcinomas expressing E-cadherin, E-cad delta 9–1 reacted with 22 tumors (13%). This new tumor marker-

monoclonal antibody system could open novel avenues for selective diagnosis and specific therapy of a subgroup of diffuse-type gastric cancer patients. (Am J Pathol 1999, 155:1803–1809)

The calcium-dependent homophilic cell adhesion molecule E-cadherin and associated catenins, cytoplasmic plaque proteins, link polarized epithelial cells and maintain the structural integrity of an epithelial monolayer. Moreover, the cadherin/catenin multiprotein complex is implicated in developmental processes and cell signaling.^{1–3} Because in carcinomas the tissue architecture is often disorganized, E-cadherin expression has been analyzed in various tumor types typically using immunohistochemistry.⁴ It could be demonstrated that E-cadherin immunoreactivity is often reduced or lost in less differentiated and invasive carcinomas.⁵ However, in diffuse-type gastric cancer, in which tumor cells generally have lost homophilic cell-to-cell contacts and invade surrounding tissues as single cells, E-cadherin immunoreactivity was detected in many cases,^{6–8} whereas in others it was found to be completely absent.⁹ The reason for this discrepancy was unknown. The selection of the cases or the use of different antibodies may, at least in part, explain these results.

Recently, E-cadherin gene mutations that may also have contributed to variable immunoreactivity have been identified in tumor cell lines, primary tumors, and lymph node metastases from gastric cancer patients.^{10–14} Remarkably, in 50% of diffuse-type gastric cancer patients E-cadherin mutations were identified that typically re-

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sulted in in-frame deletions removing partial or complete exon sequences from the extracellular portion of the transmembrane protein or point mutations resulting in amino acid substitutions.¹⁵ Complete deletion of exon 9 from the E-cadherin mRNA is a mutational hot spot in diffuse-type gastric cancer which was detected in 14% (10/70) of the patients analyzed using reverse transcription-polymerase chain reaction (RT-PCR) and sequencing.^{11–13} A variety of somatic splice-site gene mutations were identified that lead to in-frame skipping of exon 9 from the E-cadherin mRNA. Although alternative splicing mechanisms are generally possible, they have not been detected for E-cadherin and do not account for the frequent loss of exon 9. In addition, E-cadherin in-frame deletion mutations were exclusively associated with malignant tissues and never seen in nontumorous gastric epithelium from the same patients.

Because E-cadherin mRNA deletion mutations identified in primary gastric tumors and lymph node metastases do not interrupt the reading frame,¹⁵ the mutated protein may still be integrated into the plasma membrane although parts of its extracellular domain are altered. These structurally changed portions of the molecule could serve as possible targets for monoclonal antibodies. Numerous monoclonal antibodies have been generated against E-cadherin.^{16,17} However, using current antibodies, it was impossible to differentiate between normal or altered forms of the expressed E-cadherin protein. Here we report the generation and characterization of E-cad delta 9–1, a rat monoclonal antibody that specifically reacts with mutant E-cadherin lacking exon 9 and that does not recognize the wild-type protein. In a multicenter study we determined the frequency of this mutation in archival diffuse-type gastric carcinomas using E-cad delta 9–1.

Materials and Methods

Peptide Synthesis and Generation of Monoclonal Antibodies

A 13-mer peptide (Pro-Ile-Phe-Asn-Pro-Thr-Thr-Gly-Leu-Asp-Phe-Glu-Ala) was synthesized that spans the fusion junction between exon 8 and exon 10 from mutant E-cadherin lacking exon 9 and subsequently coupled to Keyhole limpet hemocyanin (KLH) using standard methods. Approximately 50 μ g of KLH-coupled peptide dissolved in phosphate buffered saline and emulsified with Freund's complete adjuvant were injected both intraperitoneally (i.p.) and subcutaneously (s.c.) into Lou/C rats. After a 4-week interval a final boost without adjuvant was given i.p. and s.c. 3 days before fusion. Fusion of the myeloma cell line P3X63-Ag8.653 with the rat immune spleen cells was performed essentially as described.¹⁸ Hybridoma supernatants were tested in a solid-phase immunoassay using bovine serum albumin-coupled peptide (10 μ g/ml) adsorbed to polystyrene microtiter plates. Solid-phase enzyme-linked immunosorbent assay (ELISA) on microtiter plates coated with mouse anti-rat Ig antibodies was used to determine the immunoglobulin

type with rat Ig class (anti-IgM; Zymed) and IgG subclass-specific mouse monoclonal antibodies.¹⁹

Cells and cDNA Transfection

Wild-type and mutant E-cadherin cDNA used for transfection were generated by RT-PCR from normal or malignant gastric tissue, respectively, and cloned into the expression vector pBAT (a gift from Prof. Takeichi; inserts are under the control of a chicken β -actin promoter). The coding regions of both cDNA constructs were confirmed by sequencing either to be unaltered (wild-type; sequence identical to the one deposited in the EMBL/GenBank database libraries, accession number Z 13009) or to contain an in-frame deletion of exon 9 as the only sequence change (exon 9 deletion). The human mammary carcinoma cell line MDA-MB-435S (ATCC) was transfected by the calcium phosphate coprecipitation method with either wild-type or mutant E-cadherin cDNA lacking exon 9 together with a plasmid for neomycin resistance. Cloning and transfection of wild-type and

Figure 1. Western blot analysis. **a:** Cell extracts from various normal tissues (liver, pancreas, colon, duodenum, stomach, esophagus, lung, kidney) were analyzed using monoclonal antibody AEC (Anti-E-Cadherin, clone 36, Transduction Lab, Lexington, KY). An extract from MDA-MB-435S mammary carcinoma cells that were transfected with wild-type E-cadherin (E-cad wt) was used as a control. AEC reacts with a protein of approximately 120 kD, corresponding to normal E-cadherin, in all lanes. **b:** The same tissues as in **a** were analyzed with the mutation-specific monoclonal antibody E-cad delta 9–1. No cross-immunoreactivity with these tissues was seen. An extract from MDA-MB-435S cells transfected with mutant E-cadherin lacking exon 9 (E-cad mut) was used as a positive control. Molecular weight in kD is indicated on the left.

Figure 2. Immunoreactivity of formalin-fixed and paraffin-embedded MDA-MB-435S cells. The cells were transfected with mutant E-cadherin lacking exon 9 (MDA-del9) or with the wild-type cDNA (MDA-wt). E-cad delta 9-1 specifically reacts with cells transfected with mutant but not with cells transfected with wild-type E-cadherin cDNA (**left panel**). Monoclonal antibody AEC reacts with cells expressing mutant and wild-type E-cadherin (**right panel**).

mutant E-cadherin cDNA has been described in detail elsewhere.²⁰

Western Blot Analysis

Extracts from E-cadherin (wild-type/mutant) transfected MDA-MB-435S cells and fresh frozen samples from liver, pancreas, colon, duodenum, stomach, esophagus, lung, and kidney were prepared according to a method previously published²¹ and used for Western blot analysis. To detect E-cadherin protein the monoclonal antibody AEC (Anti-E-Cadherin, clone 36, Transduction Laboratories, Lexington, KY) and the mutation-specific antibody E-cad delta 9-1 (clone 7E6) were used. Detection was performed using a peroxidase-coupled secondary antibody (ECL-Western, Amersham).

Immunohistochemical Analysis

To proof mutation-specific E-cad delta 9-1 immunoreactivity, 5- μ m sections of formalin-fixed and paraffin-embedded MDA-MB-435S mammary carcinoma cells transfected with either wild-type or mutant E-cadherin cDNA (see above) were analyzed. In addition, routine formalin-fixed and paraffin-embedded material from four gastric cancer patients confirmed to express mutant E-cadherin mRNA lacking exon 9 in malignant tissues¹¹ were analyzed. After microwave-based antigen retrieval with citric acid pretreatment,²² sections were incubated in 1% hydrogen peroxide for 15 minutes to block endogenous peroxidase. For detection of E-cadherin specific immunoreactivity, the specimens were incubated with either monoclonal antibody AEC (diluted 1:1000) or E-cad delta 9-1 (undiluted hybridoma supernatant) at room temperature for 2 hours. Bound antibodies were detected using the avidin-biotin-complex (ABC) peroxidase method (ABC Elite Kit, Vector, Burlingame, CA). Final staining was developed with the Sigma FAST DAB peroxidase

substrate kit (Sigma, Deisenhofen, Germany). Hemalaun was used for counterstaining. Nontumorous adult tissues (lung, heart, thyroid, placenta, salivary gland, bone marrow, lymph nodes, adipose tissue, skin, peripheral nerve, connective tissue, skeletal muscle, breast, pituitary, cerebellum, cerebral cortex, esophagus, stomach, small intestine, duodenum, colon, liver, pancreas, uterine endometrium, uterine cervix, ovary, tube, prostate, seminal vesicle, testis, epididymis) and embryonal tissues (13th week of pregnancy; umbilical cord, spinal cord, kidney, uterus, cartilage, skin, connective tissue, adipose tissue, colon, placenta, chorion) were analyzed in the same way to examine potential cross-reactivity of E-cad delta 9-1 monoclonal antibody. A series of 322 diffuse-type gastric cancer samples from Munich ($n = 97$), Düsseldorf ($n = 117$), and Tokyo ($n = 108$) were subsequently analyzed with E-cad delta 9-1. From these 322 carcinomas, 205 tumors derived from Munich and Tokyo were analyzed using both AEC and E-cad delta 9-1. All tumor sections contained nontumorous epithelium as control.

Results

E-cadherin mutation-specific monoclonal antibodies were produced in rats against a peptide that spans the fusion junction generated by direct adjoining of exon 8 and 10. Hybridoma supernatants were tested in a solid phase immunoassay using the specific peptide coupled to bovine serum albumin. Hybridoma E-cad delta 9-1 (clone 7E6) of rat IgG1 subclass was selected for further analysis.

Western Blot

To confirm specificity of the selected hybridoma and to determine potential cross-reactivity with nontumorous tissues Western blot analysis was performed. Extracts from

MDA-MB-435S mammary carcinoma cells transfected with either wild-type or exon 9-deleted E-cadherin and extracts from various fresh-frozen nontumorous tissues (liver, pancreas, colon, duodenum, stomach, esophagus, lung, kidney) were analyzed using monoclonal antibody AEC and E-cad delta 9-1. The epitope reacting with AEC is located within the intracellular domain of E-cadherin. AEC revealed a major protein band at approximately 120 kd corresponding to wild-type E-cadherin in all tissues that was also present in MDA-MB-435S cells transfected with wild-type E-cadherin (Figure 1a). Faster migrating bands most likely correspond to degradation products. No immunoreactivity in these tissues, however, was seen using E-cad delta 9-1; a protein band corresponding to mutant E-cadherin was detected only in extracts from MDA-MB-435S cells transfected with mutant E-cadherin cDNA (Figure 1b). A protein band of identical molecular weight was seen using AEC, confirming E-cadherin specificity (not shown).

Immunohistochemistry

After having demonstrated antibody specificity by Western blot we tested whether antibody E-cad delta 9-1 may react with formalin-fixed, paraffin-embedded E-cadherin transfected cells. E-cad delta 9-1 strongly stained mutant E-cadherin expressing cells, preferentially at the cell membrane, while wild-type expressing cells were not stained. In contrast, antibody AEC reacted with wild-type E-cadherin as well as with mutant E-cadherin expressing cells (Figure 2). These results indicate that the epitopes reacting with both antibodies are successfully unmasked by the procedures used.

In subsequent immunohistochemical analysis of four diffuse-type gastric carcinomas (routine formalin-fixed, paraffin-embedded material) in which the expression of mutant E-cadherin mRNA lacking exon 9 was previously demonstrated by RT-PCR and direct sequencing,¹¹ monoclonal antibody AEC reacts with nontumorous epithelial cells as well as with tumor cells in biopsies (Figure 3, a and b). However, the mutation-specific monoclonal antibody delta 9-1 exclusively labels tumor cells on serial sections from the same material, whereas nontumorous epithelial cells were not stained (Figure 3, c and d). Furthermore, tumor cells expressing mutant E-cadherin can be identified in the primary tumors (Figure 3, e and h). Because virtually all tumor cells were stained, the mutation, at least in these cases, is most likely clonal. In addition, E-cad delta 9-1 reacts strongly with tumor cells in lymphatic vessels and in lymph node metastases (not shown). Our results indicate that mutant E-cadherin protein can, for the first time, be specifically detected on tumor cells from gastric cancer patients.

To further examine potential cross-reactivity of antibody E-cad delta 9-1 with nontumorous cells, we analyzed normal tissue specimens from adults as well as from an embryo. We have not observed any cross-reactivity of E-cad delta 9-1 in the normal tissues listed in Materials and Methods.

After a rigorous demonstration that antibody E-cad delta 9-1 reacts only with mutant E-cadherin, even in archival material, the incidence of the E-cadherin variant lacking exon 9 in gastric cancer was determined. We analyzed 322 primary gastric carcinomas of the diffuse type from Germany and Japan. We found that 25 of them (8%) showed a clear reaction with delta 9-1 (Munich, 9/97; Düsseldorf, 3/117; Tokyo, 13/108). The immunoreactivity was detected mainly at the cell membrane as described above. In 205 cases immunohistochemical analysis with AEC was also performed. E-cadherin immunoreactivity with AEC was seen in 84% (172/205) of these cases. From the E-cadherin-positive tumors as determined using AEC, 13% (22/172) also reacted with E-cad delta 9-1.

Discussion

The E-cadherin exon 9 deletion variant was previously detected in 14% (10/70) of diffuse-type gastric cancer patients using RT-PCR and sequencing.¹¹⁻¹³ The generation and characterization of monoclonal antibody E-cad delta 9-1 now enabled us to easily screen a large series of gastric cancer patients specifically for the exon 9-deletion mutation. We detected 25/322 tumors expressing exon 9 deleted E-cadherin. The lower incidence of E-cad delta 9-1-positive tumors observed in the cases derived from Düsseldorf may be due to the fact that exclusively surgically R0 resected tumors were analyzed whereas in the Munich and Tokyo groups R0, R1, and R2 resected tumors were examined. The incidence of 8% is lower compared to the results using RT-PCR (14%) in a smaller series.¹¹⁻¹³ Possibly, RT-PCR is more sensitive than immunohistochemistry, or the half-life of mutant mRNA and protein may be different. Furthermore, proteins interacting with E-cadherin, catenins, may be altered, potentially resulting in degradation of mutant E-cadherin. In some cases precursor processing or posttranslational modification may be abnormal, resulting in instability of the protein without affecting mRNA stability. However, when the incidence of the mutation was related to immunoreactivity of E-cadherin, as determined by staining with AEC, a monoclonal antibody reacting with the intracellular portion of E-cadherin, we found that 13% (22/172) of the tumors expressing E-cadherin were E-cad delta 9-1-positive (the three missing cases were not included in the

Figure 3. Immunohistochemical analysis. This signet ring cell gastric carcinoma was previously confirmed to express mutant E-cadherin mRNA lacking exon 9 due to a somatic splice-site gene mutation.¹¹ **a-d:** Biopsy. Using monoclonal antibody AEC as primary antibody, both tumor cells and normal epithelial cells are stained (**a, b**); differentiation between cells expressing mutant or wild-type E-cadherin protein is not possible. In contrast, monoclonal antibody E-cad delta 9-1 exclusively stains tumor cells. Nontumorous cells, including E-cadherin-expressing epithelial cells, are not stained (**c, d**). **e-h:** Primary tumor. AEC reacts with normal epithelial cells as well as with tumor cells expressing mutant E-cadherin (**e, f**). Only tumor cells expressing abnormal E-cadherin are stained using E-cad delta 9-1 (**g, h**). Original magnifications: **a, c, e, and g**, $\times 100$; **b, d, f, and h**, $\times 200$.

series of cases stained with AEC). Because AEC reacts with E-cadherin's cytoplasmic portion, a potential cross-reactivity with another member of the cadherin superfamily showing homology to this domain, P-cadherin, is conceivable. However, using a different E-cadherin- and a P-cadherin-specific monoclonal antibody, only 1 out of 28 (4%) diffuse-type gastric carcinomas was found to express P-cadherin but not E-cadherin.⁶ Thus, E-cadherin-negative tumors that express P-cadherin seem to be rare in this type of cancer and may not significantly interfere with our approximations.

With our study E-cadherin lacking exon 9 has now been confirmed to be expressed exclusively in malignant tissues since no other tissue analyzed showed E-cad delta 9-1 immunoreactivity. The fact that this mutation is frequently found in gastric cancer patients in Europe and Japan suggests that it may play a crucial role in tumorigenesis. Data from a functional analysis using a cell culture approach are in line with this hypothesis. We could demonstrate that mutant E-cadherin has dramatic effects on calcium-dependent cell adhesion, aggregation, cell morphology, and motility, indicating that mutant E-cadherin contributes to the malignant phenotype. Interestingly, the mutation not only results in loss of E-cadherin's cell-to-cell adhesion function, but also can act in a *trans*-dominant negative manner altering the function of other cadherins, eg, N-cadherin.²⁰

Detectable immunoreactivity of E-cadherin in gastric cancer specimens using currently available antibodies may not in every case indicate the presence of a normal protein. Our mutation-specific antibody may help to resolve the discrepancy between detection of E-cadherin in the absence of homophilic cell-to-cell adhesion. Moreover, other malignant tissues besides stomach carcinoma could be identified to express mutant E-cadherin protein. E-cadherin gene mutations have been identified so far in invasive lobular breast carcinoma,²³⁻²⁵ diffuse sclerosing variant of papillary thyroid carcinoma,²⁶ and endometrial and ovarian carcinoma.²⁷ Interestingly, an exon 9 splice-site mutation was described in a breast carcinoma cell line,²⁸ although in-frame deletion mutations are rare in breast cancer.¹⁵

Mutation-specific monoclonal antibodies reacting with abnormal cell surface molecules found exclusively on tumor cells due to somatic gene mutations may open novel clinical avenues for a more specific diagnosis and therapy of gastric cancer: a humanized version of E-cad delta 9-1 could be applied to stimulate the patient's immune system or, after conjugating to radioisotopes, toxins, or drugs, it could be used to specifically target malignant cells in minimal residual cancer. With the mutation-specific monoclonal antibody E-cad delta 9-1 in hand, rapid screening of potential E-cadherin exon 9 deletion mutations in routine biopsies can now easily be performed on large populations, because this antibody works excellently with formalin-fixed, paraffin-embedded material. Cancer patients who may profit from a potential immunotherapy using modified E-cad delta 9-1 monoclonal antibody can be easily and quickly identified.

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