The Korean Journal of Pathology
2005; 39: 385-90

Expression of Epidermal Growth Factor Receptor Related Protein in Gallbladder Cancer: An Association with p53 Mutation

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Received : June 30, 2005
Accepted : July 28, 2005

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*This work was supported by the Regional Research Centers Program of the Korean Ministry of Education & Human Resources Development through the Center for Healthcare Technology Development.

Background : It has been well demonstrated that the overexpression of epidermal growth factor receptor (EGFR) is associated with numerous gastrointestinal malignancies, including gallbladder carcinoma. However, the cellular events that regulate EGFR in cancer cells have not been fully elucidated. A novel negative regulator of EGFR that is referred to as EGFR related protein (ERRP) has recently been identified. The aim of this study was to investigate the expression and localization of ERRP in gallbladder carcinoma and to examine a possible role for ERRP. **Methods :** We examined the immunohistochemical expressions of ERRP, p53 and proliferating cell nuclear antigen labeling index (PCNA-LI) in formalin-fixed, paraffin-embedded specimens of 43 cases of gallbladder carcinoma, 7 cases of adenoma and 3 cases of dysplasia. **Results :** In the normal mucosa, ERRP immunoreactivity was positive in over 64% of specimens. In contrast, the ERRP staining was positive in only 46% of the cancer specimens. The expression of ERRP in cancer cells was inversely correlated with tumor cell proliferation. The loss of ERRP expression correlated with the p53 overexpression. **Conclusions :** Our data indicate that the down-regulation or loss of ERRP could play an important role in the progression of gallbladder carcinoma. The inverse relationship between the ERRP expression and PCNA-LI suggests that ERRP may play a role in the inhibition of tumor cell proliferation in gallbladder cancer.

Key Words : Gallbladder neoplasms; Epidermal growth factor receptor; Protein p53

Epidermal growth factor receptor (EGFR) is a 170-kDa protein that consists of a cell membrane domain and a highly conserved cytoplasmic tyrosine kinase domain. Binding of EGF or transforming growth factor α (TGF-α) to the receptor activates intrinsic kinase activity, increases cytosolic calcium and ultimately stimulates the proliferation and differentiation of both normal and malignant cells.[1,2] The over-expression of EGFR with increased tyrosine kinase activity has been associated with many gastrointestinal malignancies, including gallbladder carcinoma.[2,3] A novel 1.95 kb cDNA clone, referred to as EGFR related protein (ERRP) that shows 85-90% homology to the extracellular domain of EGFR, has recently been discovered.[4] Transfection of the ERRP cDNA into the colon or prostate cancer cell lines inhibited EGFR activation and it also decreased proliferation in matrix-dependent and matrix-independent assays.[4] ERRP is a secretory protein and it may play a role in regulating gut epithelial differentiation.[5] ERRP is considered as a negative regulator of EGFR that exerts its inhibitory effect by attenuating EGFR activation.[4-6] Our previous reports showed that the down-regulation or loss of ERRP could play an important role in the differentiation and progression of gastric and pancreatic cancer.[7,8] However, the mechanism by which the loss of ERRP occurs remains unclear.

Wild-type p53 is a tumor suppressor gene product that blocks the progression of cells through the cell cycle.[9] It has been shown that p53 can either activate or suppress the activity of a number of target genes. Wild-type p53 contributes to growth inhibition by regulating, directly or indirectly, the expression of genes that are required for ongoing proliferation.[10] A mutation in the p53 gene often results in a prolonged half-life of the protein, compared to the wild type, and in the loss of functions.[11] The mutated p53 protein tends to be accumulated in the cell nuclei and this can be analyzed immunohistochemically. Therefore, positive nuclear staining suggests mutated or overexpressed p53.

Carcinoma of the gallbladder is a malignancy that generally has a poor prognosis. The increased expression and activation of EGFR have been shown to be associated with the development of gallbladder carcinoma.[3] The precise biologic role of ERRP in gallbladder carcinoma is unknown, although its inhibition of cancer cell proliferation and attenuation of EGFR phosphorylation suggest a potential role in modulating the EGFR function.[4-6] In the present study, we analyzed the expression of ERRP in gallbladder carcinoma tissues by using immunohistochemistry to determine the localization of ERRP and to evaluate the potential role of ERRP in gallbladder carcinoma. In addition, we examined the relationship between the overexpression of p53 and the expression of ERRP in gallbladder cancer.

MATERIALS AND METHODS

Materials

Both the Human Ethics Committee of Chonbuk National University Medical School and the Human Studies Subcommittee of the VA Medical Center, Long Beach, approved this study. Forty-three cases of formalin-fixed, paraffin-embedded specimens of gallbladder carcinoma tissue with the adjacent non-malignant mucosa and 7 specimens of adenoma and 3 specimens of dysplasia from surgically resected specimens were obtained from the surgical pathology archives. There were 23 male patients and 30 female patients with their ages ranging from 45 to 88 years (mean: 66.6 years). The histological type of gallbladder cancers was determined according to the World Health Organization classification.[12]

Immunohistochemistry

For ERRP and p53 immunohistochemical stainings, the immunoperoxidase method was used with the streptavidin-biotinylated horseradish peroxidase complex (DAKO, Carpinteria, CA, U.S.A.). 4 μm thick sections were cut from the formalin-fixed paraffin-embedded tissue blocks. After deparaffinization, they were incubated in methanol that contained 0.3% hydrogen peroxide at room temperature for 20 min to block the endogenous peroxidase and the sections were treated with pepsin for 10 min at room temperature (for ERRP staining), and then they were treated with a microwave antigen retrieval procedure in 0.01 M sodium citrate buffer for 10 min (for p53 immunostaining). After blocking the endogenous biotin, the sections were incubated with Protein Block Serum-Free media (DAKO, Carpinteria, CA) at room temperature for 10 min to block any nonspecific staining, and then they were incubated for 1 h at room temperature with anti-ERRP antibody or anti-p53 antibody (DAKO, Carpinteria, CA). Polyclonal antibodies to ERRP were generated in rabbits against the "U" region of ERRP; this region is comprised of 27 amino acids and it has no homology with any known sequence in the current database.[4] On western-blot analysis, the antibodies reacted strongly with a 55 kD protein that corresponds to the calculated molecular mass of ERRP, of which the open reading frame is composed of 479 amino acids. No cross reactivity of ERRP with EGFR or any of its family members was noted.[5] After washing, the sections were incubated with a biotin-conjugated secondary antibody at room temperature for 30 min and they were finally incubated with peroxidase conjugated streptavidin at room temperature for 30 min. Peroxidase activity was detected with the enzyme substrate 3 amino-9-ethyl carbazole. Samples with immunopositivity at least 10% of the tumor cells were defined as positive cases.

Proliferating cell nuclear antigen

To determine the relationship between the ERRP expression and the proliferation activity of cancer cells, we performed doubleimmuno staining for proliferating cell nuclear antigen (PCNA), which is a nuclear antigen that is present only in proliferating cells, using the Envision™ Doublestain System (DAKO, Carpinteria, CA). The tissue sections were deparaffinized and the slides were treated with a microwave antigen retrieval procedure in 0.01 M sodium citrate buffer for 10 min (Envision™ Doublestain System step 1). After quenching of the endogenous peroxidase activity with peroxidase blocking reagent (DAKO, Carpinteria, CA), the sections were incubated with the anti-PCNA antibody (DAKO, Carpinteria, CA) for 1 h at room temperature (Envision™ Doublestain System step 2). The slides were rinsed with

washing buffer and then incubated with labeled polymer-horseradish peroxidase-anti mouse and anti rabbit antibodies (Envision™ Doublestain System step 3) for 30 min at room temperature. The peroxidase activity was detected with the enzyme substrate 3,3'-diaminobenzidine tetrachloride (Envision™ Doublestain System step 4). After quenching the enzyme reaction, the slides were incubated in Doublestain Block (Envision™ Doublestain System step 5) at room temperature for 5 min to block endogenous phosphatase. Thereafter, the slides were incubated with anti-ERRP antibody for 1 h at room temperature (Envision™ Doublestain System step 6). After washing, the slides were incubated with labeled polymer-alkaline phosphatase-anti mouse and anti rabbit antibody (Envision™ Doublestain System step 7) for 30 min at room temperature. Fast red solution was used for the localization of the antibody. The sections were counterstained with Mayer's hematoxylin. To study the role of ERRP in tumor cell proliferation, we calculated the PCNA positive cancer cells/ERRP negative cancer cells and the PCNA positive cancer cells/ERRP positive cancer cells, respectively. The PCNA labeling index (PCNA-LI) was defined as the percentage of nuclei with positive PCNA staining.

Statistical analysis

The values are expressed as means±SE. Student's t-test was used to determine the differences of PCNA-LI between the two groups. The association between the expression of ERRP and the p53 protein over-expression was tested for by the chi-square test. A p value of <0.05 was considered statistically significant.

RESULTS

Forty-three gallbladder cancer specimens included 8 cases of carcinoma in situ, 29 cases of invasive adenocarcinomas, 2 cases of clear cell adenocarcinomas, 2 cases of adenosquamous carcinomas,

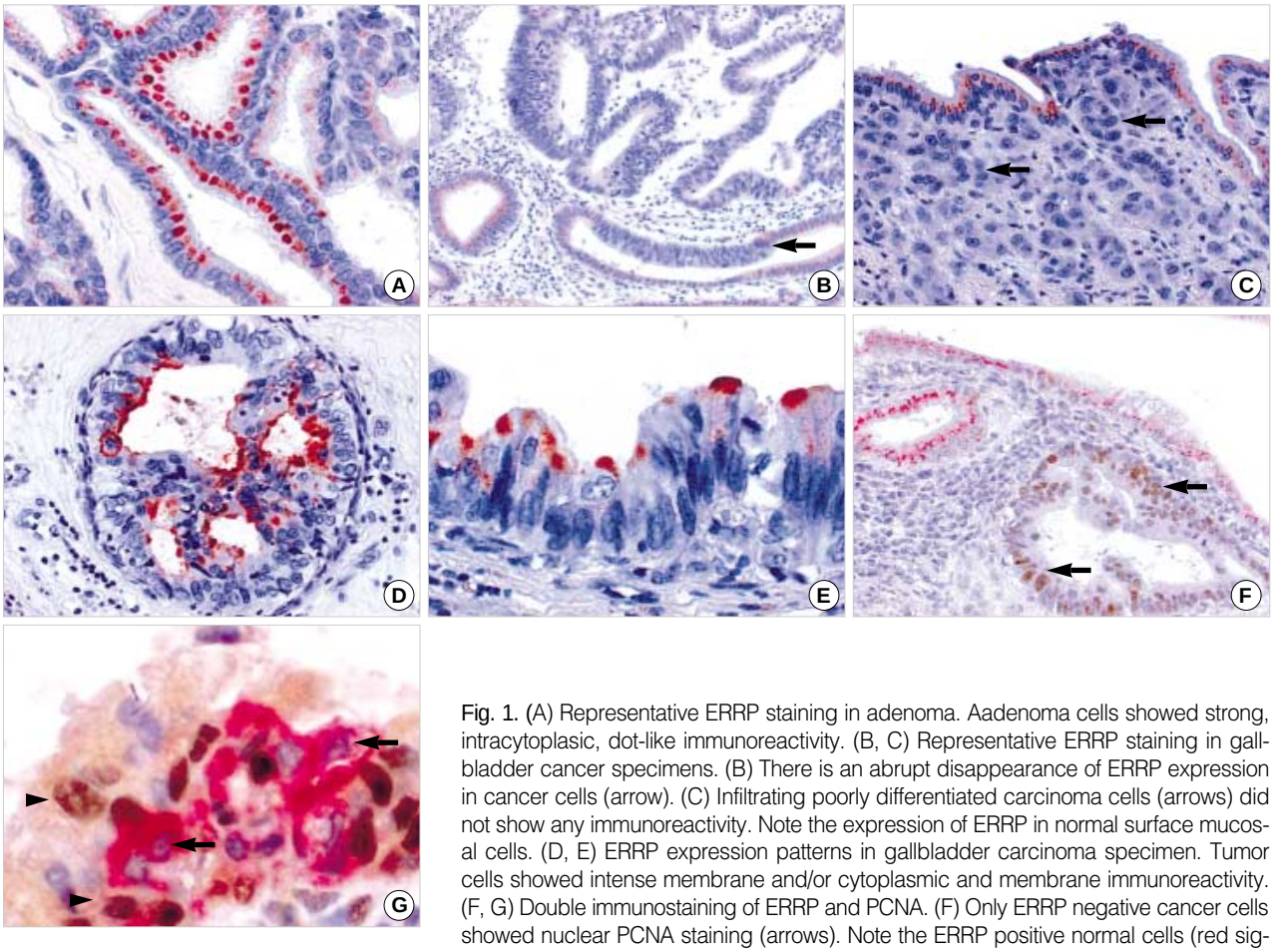

Fig. 1. (A) Representative ERRP staining in adenoma. Aadenoma cells showed strong, intracytoplasic, dot-like immunoreactivity. (B, C) Representative ERRP staining in gallbladder cancer specimens. (B) There is an abrupt disappearance of ERRP expression in cancer cells (arrow). (C) Infiltrating poorly differentiated carcinoma cells (arrows) did not show any immunoreactivity. Note the expression of ERRP in normal surface mucosal cells. (D, E) ERRP expression patterns in gallbladder carcinoma specimen. Tumor cells showed intense membrane and/or cytoplasmic and membrane immunoreactivity. (F, G) Double immunostaining of ERRP and PCNA. (F) Only ERRP negative cancer cells showed nuclear PCNA staining (arrows). Note the ERRP positive normal cells (red signals) did not show immunoreactivity for PCNA. (G) ERRP positive carcinoma cells (arrows, red signals) are distinguished from adjacent PCNA positive carcinoma cells (arrowheads), which showed faint or negative staining for ERRP.

1 case of mucinous adenocarcinoma and 1 case of signet ring cell carcinoma. According to the tumor cell differentiation, 14 cases were grade I, 19 cases were grade II and 4 cases were grade III. ERRP expression was detected in 29 (64%) of 45 non-tumorous mucosa areas. The ERRP expression was exclusively confined to the epithelial cells, and it was absent in the mesenchymal cells. In the normal mucosa, distinct perinuclear, cytoplasmic, dot-like ERRP expression was present in the superficial epithelium and in some glandular cells. The expression of ERRP was detected in 4 (57%) of 7 adenomas and 2 (67%) of 3 dysplasia samples in a distinct perinuclear staining pattern that was restricted to the cytoplasm of the epithelial cells. ERRP expressions in adenoma and dysplasia were stronger in intensity than those in the normal mucosa (Fig. 1A). In the gallbladder carcinoma, there was a marked loss of ERRP staining throughout the tumor (Fig. 1B, C). Only 20 (46%) of 43 carcinoma specimens showed positive staining and many of these positive specimens showed only a focal reaction. In the ERRP positive cancer specimens, two staining patterns were present in the cancer cells; one was strong staining of the cell membranous, and the other was dense membranous and cytoplasmic staining with a loss of the polarized distribution pattern that was present in the normal mucosa. Some of tumor cells revealed the luminal surface expression of ERRP, and this suggested secretory activity (Fig. 1D, E).

Double immunostaining for ERRP and PCNA showed that the expression of ERRP was negatively correlated with the PCNA expression. The ERRP positive carcinoma cells exhibited only a very faint or no PCNA immunoreactivity. This pattern clearly distinguished the ERRP positive cells from the adjacent PCNA positive cells (Fig. 1F, G). The PCNA-LI was significantly lower in the ERRP positive cells (24.2±1.4%) than in the ERRP negative cells (49.7±2.6%) ($p<0.001$).

p53 overexpression was observed in 33 (76%) of 43 cancers. The staining was nuclear in all the positive cases. One (14%) of 7 adenomas and 1 (33%) of 3 dysplasia specimens showed p53 over-expression. There was a significant correlation between the loss of ERRP expression and p53 overexpression in the 53 gallbladder specimens that were examined (Table 1) ($p=0.015$).

Table 1. Relationship between expressions of ERRP and p53 in 43 cases of carcinoma, 7 cases of adenoma and 3 cases of dysplasia of the gallbladder

ERRP expression	p53 positive	p53 negative	Total
Positive	13	13	26
Negative	22	5	27
Total	35	18	53

ERRP, epidermal growth factor receptor related protein.

DISCUSSION

This study demonstrated for the first time that (1) ERRP is down regulated in gallbladder carcinoma, (2) there is a clear spatial difference of ERRP immunoreactivity between in the normal cells, where it was localized to the cytoplasm as a perinuclear, dot-like pattern, and in the cancer cells, where strong membrane and/or cytoplasmic staining was observed, (3) the expression of ERRP in cancer cells is inversely correlated with cancer cell proliferation, and (4) the loss of ERRP expression is significantly correlated with p53 overexpression. These data indicate that the loss of ERRP expression could play an important role in the progression of gallbladder cancer.

Double immunostaining for ERRP and PCNA in the normal mucosa showed that the ERRP positive cells exhibited only a very faint or no PCNA immunoreactivity. These findings suggest that ERRP may be constitutively expressed at a low level in normal resting cells and it has an anti-proliferative role in the normal physiologic condition. We demonstrated that there were some enhancement of the ERRP signal in the adenoma and dysplasia specimens, and that the loss of ERRP expression correlated with the development of gallbladder carcinoma. The observed increase of cytoplasmic ERRP staining in some of the adenoma and dysplasia specimens suggests that this is an early change in the transition to the neoplastic process. The exact function of cytoplasmic ERRP in the non-malignant cells remains to be elucidated.

Previous reports have demonstrated that the ERRP levels substantially increased in a time dependent fashion during the differentiation of Caco-2 cells.[5] Similarly, ERRP expression correlated with the differentiation of pancreatic and gastric adenocarcinoma.[5,7,8] Our recent study demonstrated that the expression of ERRP in hepatocellular carcinoma cells was inversely correlated with the proliferation activity and tumor size.[13] These observations support the hypothesis that ERRP is an important determinant of epithelial cancer differentiation and development.

We demonstrated the increased signal and strong membranous staining pattern of ERRP in the ERRP positive carcinoma specimens, where it was localized to the cytoplasm as perinuclear staining in the non-tumorous cells. The significance of the change of ERRP localization to the membrane in carcinoma cells is uncertain. Three splice variants of the EGFR gene that encode the extracellular domain of the receptor have been isolated.[14-16] Accumulating evidences have indicated that the truncated EGFR containing the EGFR-binding site, but not the kinase domain, is associated with an inhibition of cancer cell

growth,[17,18] and the regulation of EGFR kinase may be due to the specific interaction with the ligand-binding domain of the EGF receptor kinase.[18] Transfection of ERRP cDNA into colon or prostate cancer cell lines not only inhibited EGFR activation, but it also significantly decreased the proliferation of cancer cells.[4] Moreover, we reported that the EGF-induced activation of EGFR in gastric cancer cells was markedly inhibited by affinity purified ERRP.[7] In the present study, we found an intense selective membrane ERRP staining pattern in carcinoma cells, and that these ERRP positive cancer cells had significantly decreased PCNA-LI compared to the ERRP negative cancer cells. Although the ERRP is not the product of the primary EGFR transcript, it showed 85-90% homology to the extracellular domain of EGFR.[4] Considering that the functional form of EGFR could be localized on the plasma membrane, the enhanced expression of immunoreactive ERRP on the surface of tumor cells demonstrated a possible role of ERRP in the negative regulation of EGFR activation as well as in tumor cell proliferation. Taken together, these data suggest two possible mechanisms regarding the inhibition of phosphorylation of EGFR by ERRP. If ERRP is capable of binding to ligands, it can modulate the activity of the transmembrane receptor by binding to other ligands.[15] Regardless of the ligand-binding ability, ERRP may form an inactive heterodimer with EGFR and it may inhibit the phosphorylation of EGFR. Indeed, a soluble EGFR from A431 cells has been demonstrated to inhibit the tyrosine kinase activity of the transmembrane receptor through the direct interaction of the soluble receptor with the EGFR.[15,18] Our recent study demonstrated that reduced EGFR phosphorylation was partly due to the sequestration of EGFR ligands by ERRP, which resulted in the formation of inactive heterodimers with EGFR, and this further supports our contention.[6] However, the exact mechanism by which ERRP inhibits EGFR activation remains to be elucidated.

p53 is the most frequently mutated gene in human cancer, including gallbladder cancer.[19] Tumor suppressor p53 is a nuclear transcription factor that blocks cell cycle progression and induces apoptosis. p53 mutation leads to the inactivation of p53 function through the abolition of p53-specific DNA binding and transactivation. Loss of its function appears to confer selective advantages to cells via deregulated growth and resistance to cell death.[9-11,20] In the present study, the loss of ERRP expression was significantly related to p53 overexpression. Previous studies have indicated significant positive correlation between PCNA-LI and p53 immunoreactivity in gastrointestinal cancer tissue.[21,22] A mutation in the p53 gene often results in a prolonged half-life of the protein. Therefore, the positive nuclear immunoreactivity suggests mutated or overexpressed p53. These findings suggest that the expression loss of ERRP, a novel negative regulator of EGFR, could partly be the result of p53 mutation.

In conclusion, our data indicate that the down-regulation of ERRP is associated with the development of gallbladder carcinoma. The inverse relation between ERRP expression and PCNA-LI suggests that ERRP may play a role in the inhibition of tumor cell proliferation. The correlation between p53 overexpression, as detected by immunohistochemistry, and the loss of ERRP expression suggests that p53 mutation may be partly responsible for the loss of ERRP expression in gallbladder carcinoma.

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