Overexpression of epithelial cell transforming 2 protein in colorectal carcinoma predicts a poor prognosis

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Abstract. Epithelial cell transforming 2 (Ect2) protein is a member of the human diffuse B-cell lymphoma family of guanine nucleotide exchange factors, which activate the Ras homolog gene family of small GTPases; however, the clinical implications of Ect2 in colorectal carcinoma (CRC) are unclear. The present study aimed to determine the relationship between Ect2 expression and prognosis in patients with CRC. Western blot analysis and immunohistochemistry assays were used to determine the expression of Ect2 in CRC and paired non-cancerous tissues from 66 patients. The correlation between Ect2 expression and clinicopathological parameters was assessed using χ2 tests. Patient survival was determined using the Kaplan-Meier method and log-rank test. Cox regression was used for multivariate analysis of prognostic factors. Results demonstrated that Ect2 protein was highly expressed in human CRC samples [29/45 (64.45%)] and significantly correlated with a poor prognosis (P<0.05). Compared with normal tissues, CRC tissues demonstrated higher expression levels of Ect2 mRNA [44/66 (66.67%)]. In addition, highly-expressed Ect2 was significantly associated with recurrence (P=0.023) and invasion (P=0.008) of CRC. High Ect2 expression levels in patients were associated with poorer overall survival (OS) and disease-free survival (DFS) compared with lower expression levels of Ect2. Based on multivariate analysis, Ect2 overexpression was significantly correlated with OS and DFS (P=0.015 and 0.020, respectively). In conclusion, Ect2 overexpression is an independent and important prognostic factor for OS and DFS in patients with CRC.

Introduction

The incidence of colorectal cancer (CRC) varies worldwide, with the incidence of CRC being higher in North America, Australia, northern and western Europe compared with other regions. CRC is less prevalent in developing countries, particularly in Africa and Asia (1). Over 90% of CRC cases occur in people ≥50 years of age (2). Nevertheless, CRC incidence appears to be increasing amongst the younger population and the incidence of CRC in individuals of ≥40 years of age ranges from 1.6 to 23% (3,4). The lowest incidences of CRC are in Asia and countries in the Middle East, and the highest incidences of CRC are in Europe and the United States. The incidence of CRC has not been established in north Africa. Therefore, in order to improve the survival rate in patients with CRC, there is an urgent requirement to identify putative diagnostic markers, prognostic factors and treatment strategies.

Through the activation of the Ras homolog gene family member A (RhoA) by guanine nucleotide exchange factor (GEF) and epithelial cell transforming 2 (Ect2), the central spindle stimulates contractile ring formation (5). The central spindle complex is composed of a heterotetramer of mitotic kinesin-like protein 1 and MgcRacGAP/Cyk-4 (6,7) and helps to form the central spindle during anaphase. Ect2 binds to Cyk-4 via N-terminal breast cancer 1 C-terminus domains, which recruits Ect2 to the central spindle (8-10). The GEF activity of Ect2 is mediated by the conserved Dbl homology (DH) and pleckstrin homology (PH) domains in the C-terminus (11). The DH domain catalyzes nucleotide exchange on RhoA and the PH domain is involved in cortical localization of Ect2, although the molecular function, including phospholipid or protein interactions, is not known (11-14). In metaphase, cyclin dependent kinase 1 phosphorylation induces a conformational change in Ect2, which prevents Cyk-4 binding and inhibits GEF activity (9,13). Despite the knowledge of the involvement of Ect2 in the cell cycle, the importance of the association between Ect2 expression levels and CRC tumor diagnosis, prognosis and clinical and pathologic features remains unclear.

The present study analyzed the expression levels of Ect2 in CRC and non-cancerous tissues using western blot analysis and immunohistochemistry. Furthermore, the...
relationship between Ect2 overexpression and clinicopathological features and post-resection survival was determined. The results of the present study demonstrated that Ect2 may be used as an independent prognostic factor in patients with CRC and may also be used as a therapeutic target for CRC.

Materials and methods

Ethics statement. Written informed consent was provided by all patients enrolled in the present study. The study was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University (Shenyang, China).

Patients. CRC and paired non-cancerous tissues were collected from 66 patients (38 males and 28 females) who had undergone hepatectomies for CRC at The First Affiliated Hospital of China Medical University and 202 Hospital of People’s Liberation Army (both Shenyang, China) between July 2007 and July 2012. Histopathological analyses were performed independently by pathologists from both hospitals. The median age of the patients was 53 years (range, 37–81 years). The gender, age and clinicopathological features of patients, including tumor size, lymph node metastasis status, tumor differentiation, complication, number of tumors, histopathological differentiation, recurrence, invasion status and TNM staging are summarized in Table I. TNM staging was determined using the United Network of Organ Sharing-modified TNM staging system for CRC (15). Tumor differentiation was based on the World Health Organization criteria (16).

Western blot (WB) analysis. Total protein was extracted from 66 pairs of fresh tissue samples of CRC and paired non-cancerous tissues. Cells were lysed in pre-chilled RIPA lysis buffer (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) for 30 min. Samples were centrifuged at 15,000 x g for 20 min to obtain the supernatant. Following this, protein samples (30 µg/well) were separated by 10% SDS-PAGE and transferred onto a 0.45-mm nitrocellulose membrane. Subsequently, the membrane was blocked at 25˚C overnight with a blocking buffer (pH 7.6) containing 5% non-fat milk prior to incubation with primary rabbit anti-human Ect2 polyclonal antibody (1:300, 1 h at room temperature; sc-69879; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was performed using 500 µg total RNA from each sample. qPCR was performed using a SYBR Green PCR master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and a Rotor Gene 6000 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The qPCR cycling conditions were as follows: 30 sec at 95˚C, followed by two-step PCR for 40 cycles of 95˚C for 5 sec, 60˚C for 60 sec and 85˚C for 5 sec. Each reaction mixture contained 2 µl cDNA sample, 1 µl of each of the forward and reverse primers (Applied Biosystems; Thermo Fisher Scientific, Inc.), 8.5 µl RNase-free H₂O and 12.5 µl SYBR Green, amounting to a total reaction volume of 25 µl. β-actin was used as a reference for gene normalization. The sequences of the primers used were as follows: Ect2 forward, 5’-TCCCTCCGGTGACAGACAG-3’, Ect2 reverse, 5’-GCTTTGAAAAATGGGACT-3’; and β-actin forward, 5’-ATAGCTAGCAGGCCTGGATAGCAAGTCG-3’, β-actin reverse, 5’-CACCTTCTAAAAATGCAGTGCTGTCG-3’. The relative levels of gene expression were determined using the 2-ΔΔCq method (19). Experiments were repeated three times.

Immunohistochemistry (IHC). Ect2 expression was examined using immunohistochemistry on paraffin-embedded samples from 66 patients with CRC. Tumor samples were fixed in 10% formalin prior to embedding the samples in paraffin. Following this, the embedded samples were cut into 5-µm consecutive sections. After general deparaffinization,
antigen retrieval was implemented with an autoclave using 0.01 mol/l sodium citrate buffer (pH 6.0) for 30 sec. H₂O₂, (0.3%) was added to samples to block endogenous peroxidase activity for 30 min at 37°C. Non-specific immunoglobulin binding sites were blocked by incubating the samples with normal goat serum (ZSBG-Bio Company) for 30 min at 37°C. Following this, sections were incubated at 4°C overnight with a purified primary Ect2 rabbit polyclonal antibody (1:150; NB100-74663; Novus Biologicals, LLC, Littleton, CO, USA). After three 5-min washes with phosphate buffered saline, a secondary biotinylated anti-rabbit immunoglobulin G (IgG) (NC-100, 1:300) or anti-mouse IgG antibody (NC-1390, 1:400) (both Fuzhou MaiXin Biotech Co., Ltd., Fuzhou, China) was applied to the sections for 30 min at room temperature. After washing with PBS three times for 5 min each, streptavidin-biotin conjugated with horseradish peroxidase (Reagent C of SP-9000, ZSBG-Bio Company) was applied to the sections for 30 min at room temperature, and the slides were colored with 3,3'-diaminobenzidine tetrahydrochloride. The sections were then stained with Meyer's hematoxylin. Normal rabbit/mouse IgG was used as the primary antibody at the same dilution as a negative control.

Follow-up. A total of 66 patients were available for follow-up, with follow-up time ranging from 3-53 months (median, 26 months) after experiment initiation. On 31st May 2012 (the census date), 45 (68.18%) patients were alive and 21 (31.82%) patients had succumbed to their disease.

Statistical analysis. SPSS v.17.0 software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. The relationships between tumor markers and other parameters were evaluated using t test and a χ² test. Patient survival was analyzed using the Kaplan-Meier method and the log-rank test was used to analyze survival differences. A Cox regression model was used for univariate and multivariate analysis of prognostic parameters. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of Ect2 mRNA in clinical tissue specimens. Ect2 mRNA expression levels in clinical samples were determined using RT-qPCR. Of the 66 patients, 44 (66.67%) demonstrated a higher level of Ect2 mRNA in CRC tissue than in the paired non-cancerous tissue (Fig. 1). The mean level of Ect2 mRNA expression in CRC tissues (mean ± standard deviation, 41.75±35.10; standardized by β-actin gene expression) was significantly higher compared with the level (19.21±20.03) in the paired non-cancerous tissues (t=4.196, P<0.01; Fig. 1).

WB and IHC. WB was used to evaluate Ect2 protein expression levels in CRC and paired non-cancerous tissues from 66 patients and expression levels were normalized to β-actin (Fig. 2). Ect2 protein was detected by IHC in 45 CRC specimens. Overexpression of Ect2 was observed in ~two thirds of tumor samples [29 of 45 (64.45%); 2-3+] The remaining 16 cases (35.5%; Table I) demonstrated low levels of Ect2 protein expression (0 to 1+). Ect2 protein expression was observed in the nucleus and cytoplasm of tumor cells at variable levels (Fig. 3). The Ect2 staining scores were significantly influenced by recurrence and vein invasion (Table I; P=0.022 and 0.008, respectively); however, other clinicopathological factors did not significantly influence Ect2 staining scores (Table I; P>0.05).

Influence of Ect2 expression levels on overall survival (OS) and disease-free survival (DFS) in CRC. According to univariate analysis, gender, tumor size, age, differentiation, histopathological type and family history were not predictive for DFS or OS (Table II; P>0.05). Invasion and TNM stage were significant predictors for DFS (P<0.001 and P=0.009, respectively). Ect2, TNM stage, complication and invasion were demonstrated to be significant independent prognostic factors for OS (Table II; P=0.004, 0.007, 0.007 and P<0.001, respectively). Multivariate analysis demonstrated that Ect2 expression levels [hazard ratio (HR)=1.745; 95% confidence interval (CI), 1.121-2.574; P=0.015], complication (HR=1.695; 95% CI, 1.079-2.525; P=0.019) and invasion (HR=2.654; 95% CI, 1.452-3.738; P<0.001) were significant independent prognostic factors for OS in patients with CRC (Table III). Furthermore, Ect2 expression levels (HR=1.671; 95% CI, 1.093-2.424; P=0.020), TNM stage (HR=1.633; 95% CI, 1.216-2.284; P=0.038) and invasion (HR=2.710; 95% CI, 1.913-4.346; P<0.001) were significant independent prognostic factors for DFS in patients with CRC (Table III). According to the Kaplan-Meier method and log-rank test, CRC specimens with a higher level of Ect2 were demonstrated to have significantly shorter OS or DFS (log-rank value=10.54 and 8.20; P=0.007 and 0.003, respectively; Fig. 4).

Discussion

Ect2, a Rho GEF, is a proto-oncogene that has transforming ability in fibroblasts and is involved in cytokinesis (13,20). Ect2 has been reported to be expressed at elevated levels in various types of cancer, including esophageal cancer, lung cancer, and glioma (21,22). A study by Chalamalasetty et al (12) demonstrated that Ect2 is localized to the central spindle and the cell cortex in mitotic cells. Depletion of Ect2 impairs cleavage furrow formation and inhibits the accumulation of RhoA and citron kinase at the cleavage furrow, suggesting that Ect2 is essential for cytokinesis (23). A study by Hirata et al (21) using tumor tissue microarray analysis indicated that a high Ect2 expression levels are associated with poor prognosis for patients with non-small cell lung cancer. Furthermore, the study by Hirata et al (21) also demonstrated that Ect2 knockdown by small interfering RNA was able to effectively suppress lung cancer cell growth, suggesting a specific role for Ect2 in lung cancer development. Interestingly, Ect2 protein expression levels were significantly upregulated in the lungs of a murine model of pulmonary squamous cancer, suggesting that increased Ect2 expression contributes to lung tumor development (24). Thus, Ect2 is an independent factor that may affect patient prognosis; however, it has not been determined if factors of this type are prognostic of CRC. Further studies
LI et al: OVEREXPRESSION OF Ect2 IN COLORECTAL CARCINOMA PREDICTS A POOR PROGNOSIS

In the present study, IHC revealed that there was a significant association between elevated Ect2 expression levels and tumor invasion and recurrence in the 66 CRC specimens examined according to $\chi^2$ tests; however, there was no significant relationship with other clinicopathological parameters of CRC, including age, gender, tumor size, TNM stage, lymph node metastasis status, tumor differentiation, complication and number of tumors. These findings therefore suggest that Ect2 expression level is relative to CRC invasion and recurrence, and that Ect2 may have an important role in tumor carcinogenesis and CRC progression. Furthermore, patients who overexpressed Ect2 had a lower DFS and OS following surgery than those who demonstrated reduced Ect2 expression levels, according to Kaplan-Meier analysis. Multivariate Cox regression analysis indicated that, among the variables analyzed, elevated Ect2 expression was an independent prognostic factor for DFS and OS, without new recurrent tumors. According to the data obtained, the results indicated that high Ect2 expression levels may be associated with a poor prognosis, which also suggests that Ect2 may be a novel independent prognostic indicator in patients with CRC. Based on the staining results, it was demonstrated that Ect2 may be important in predicting CRC recurrence; however, the mechanism involved is unclear. A study by

| Characteristic | Number | Ect2 expression level | $\chi^2$ | P-value |
|---------------|--------|----------------------|--------|---------|
|               |        | High | Low |
| Total cases  | 66     | 44   | 22  |
| Age (years)  |        |      |     |
| $\geq$60     | 40     | 25   | 15  | 0.793   | 0.373   |
| $<60$        | 26     | 19   | 7   |
| Gender       |        |      |     |
| Male         | 37     | 27   | 10  | 1.507   | 0.220   |
| Female       | 29     | 17   | 12  |
| Tumor size (cm) |        |      |     |
| $\geq$5      | 39     | 28   | 11  | 1.128   | 0.288   |
| $<5$         | 27     | 16   | 11  |
| TNM stage    |        |      |     |
| I+II         | 30     | 18   | 12  | 1.100   | 0.294   |
| III+IV       | 36     | 26   | 10  |
| Lymph node metastasis status |        |      |     |
| Positive     | 38     | 28   | 10  | 1.985   | 0.159   |
| Negative     | 28     | 16   | 12  |
| Tumor differentiation |        |      |     |
| WD           | 27     | 19   | 8   | 0.354   | 0.838   |
| MD           | 24     | 15   | 9   |
| PD           | 15     | 10   | 5   |
| Complications |        |      |     |
| Yes          | 35     | 20   | 15  | 3.041   | 0.081   |
| No           | 31     | 24   | 7   |
| Number of tumors |        |      |     |
| Single       | 36     | 23   | 13  | 0.275   | 0.600   |
| More         | 30     | 21   | 9   |
| Recurrence   |        |      |     |
| Yes          | 37     | 29   | 8   | 5.198   | 0.023   |
| No           | 29     | 15   | 14  |
| Invasion     |        |      |     |
| Yes          | 39     | 31   | 8   | 7.051   | 0.008   |
| No           | 27     | 13   | 14  |

Ect2, epithelial cell transforming 2; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated.
Hayashi et al (25) reported that recurrence of CRC in patients was associated with poor prognosis. It is possible to hypothesize that when Ect2 expression is stable, dephosphorylation of Ect2 is inhibited, which may promote the recurrence of CRC; however, the signal pathway in which the coding gene has been changed is unknown.

In conclusion, the present study demonstrated that Ect2 is overexpressed in a great proportion of CRC cases, and that high Ect2 expression levels are associated with the poor results in CRC post resection. Therefore, Ect2 may be applied as an independent biomarker to examine increased risk of recurrence. Furthermore, Ect2 overexpression has been
LI et al: OVEREXPRESSION OF Ect2 IN COLORECTAL CARCINOMA PREDICTS A POOR PROGNOSIS

4867

Demonstrated to be an independent prognostic indicator in CRC and Ect2 may therefore be a novel molecular biomarker in the diagnosis and prognosis, or a therapeutic target, of this deadly disease.

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