International Journal of Oral Biology, Vol. 31, No. 2, June 30 2006, p. 27~32

International Journal of Oral Biology

Methylation Status and Expression of E-cadherin in Oral Squamous Cell Carcinomas Compared to Benign Oral Epithelial Lesions

Hyun-Jin Son[1], Jung-Youb Chu[1], Eui-Sic Cho[1], Dong-Geun Lee[2], Myung-Gee Min[1], Suk-Keun Lee[3], and Nam-Pyo Cho[1]

[1]*Institute of Oral Bioscience, School of Dentistry, Chonbuk National University, Jeonju, Korea*
[2]*Department of Pathology, School of Medicine, Chonbuk National University, Jeonju, Korea*
[3]*Department of Oral Pathology, College of Dentistry, Kangnung National University, Kangnung, Korea*

(Received February 28, 2006 ; Accepted March 20, 2006)

Expression of invasion/metastasis suppressor, E-cadherin, is reduced in many types of human carcinomas. Although somatic and germline mutations in the *CDH1*, which encodes the human E-cadherin, have frequently been reported in cases with diffuse gastric and lobular breast cancers, irreversible genetic inactivations are rare in other human carcinomas. Recently, it has been well documented that some genes in human cancers may be inactivated by altered CpG methylation. Herein, we determined the expression and methylation status of E-cadherin in oral squamous cell carcinoma (SCC) by immunohistochemistry and methylation-specific PCR. The expression of E-cadherin was significantly higher in the well-differentiated oral SCCs than the moderately or poorly differentiated ones. None of eight tested benign epithelial hyperplasias showed aberrant methylation, whereas five of 12 oral squamous cell carcinomas showed aberrant methylation. When we compared E-cadherin expression with methylation status, oral SCCs with normal methylation showed a higher expression of E-cadherin than those with methylation. These findings suggest that aberrant CpG methylation of *CDH1* promoter region is closely associated with transcriptional inactivation and might be involved in tumor progression of the oral mucosa.

Keywords: CDH1, E-Cadherin, CpG Methylation, Oral Squamous Cell Carcinoma.

*Corresponding author: Nam-Pyo Cho, Institute of Oral Bioscience, School of Dentistry, Chonbuk National University, 664-14, Duckjin-dong, Jeonju, 561-756, Republic of Korea. Tel.: +82-63-270-4026; FAX.: +82-63-270-4004; E-mail: npcho@moak.chonbuk.ac.kr

Introduction

Squamous cell carcinoma (SCC) is the most common malignant tumor of the oral mucosa, comprising more than 90% of intraoral cancers (Sciubba, 2003). Even though the epidemiology has been well described, the molecular steps involved in the pathogenesis of these common neoplasms are poorly understood.

E-cadherin, a calcium-dependent transmembrane adhesion molecule, plays a key role in the establishment and maintenance of the normal epithelial phenotype (Takeichi, 2003). Several studies have suggested that a loss of E-cadherin may be associated with tumor progression in epidermal carcinogenesis, and E-cadherin in particular acts as a suppressor of the invasive ability or metastatic phenotype (Schipper *et al.*, 1991; Chen *et al.*, 2003). However, to our knowledge, the mechanisms responsible for the reduction or absence of E-cadherin in oral SCCs are poorly understood. Although somatic and germline mutations in the E-cadherin gene (*CDH1*) have frequently been reported in cases with diffuse gastric and lobular breast cancers (Humar *et al.*, 2002; Lei *et al.*, 2002), irreversible genetic inactivation is rare in other human carcinomas. Recently, it has been suggested that the *CDH1* may be inactivated by CpG methylation in a wide variety of carcinomas of various origins, including the breast, and lung (Sarrio *et al.*, 2003; Topaloglu *et al.*, 2004).

Therefore, this study evaluated the CpG methylation status around the *CDH1* promoter region by methylation-specific PCR (MSP) and expression of E-cadherin by quantitative immunohistochemical assay using an image analyzing system, in order to establish its role in the carcinogenesis of

oral SCC compared with benign oral epithelial hyperplasia.

Materials and methods

Thirty-six cases of oral SCC and 33 cases of non-neoplastic oral epithelial hyperplasia were collected from the 1995-2000 pathological files of Chonbuk National University Hospital and examined by light microscopy using the sections stained with H&E in order to assess the proportion of tumor tissue and the degree of differentiation. SCC samples were histologically classified according to the Broders' grading (Broders, 1941) and clinically staged by American Joint Committee on Cancer staging system (Beahrs *et al.*, 1992). Moderately- and poorly-differentiated tumors were grouped for comparison with the well-differentiated ones. Of the 36 SCCs, 21 cases were well differentiated and the remaining 15 cases were moderately- or poorly-differentiated. The clinical data was obtained from 22 cases by reviewing the patient records. Ten cases belonged to stage I or II and 12 cases belonged to stage III or IV. The tumors not treated with either radiation or chemotherapy were included in this study.

Immunohistochemistry

Immunohistochemical staining was carried out using a CSA system (Dako, Carpinteria, CA), which is based on the peroxidase-catalysed deposition of biotinylated tyramide. Briefly, 4 μm sections were mounted onto silanized slides, dewaxed in xylene, and then rehydrated with ethanol. The sections were immersed in a 0.1 M citrate buffer solution at pH 6.0. After boiling in a microwave oven for 5 min, the slides were cooled to room temperature and then rinsed in phosphate buffered saline (pH 7.4) for 10 min. In order to quench the endogenous peroxidase activity, the specimens were treated with 3% hydrogen peroxide for 5 min at room temperature. The sections were incubated in an anti-human E-cadherin monoclonal antibody (HECD-1, Zymed, CA; diluted 1:200), and then treated with four sequential 15 min incubations with the biotinylated link antibody, streptavidin-biotin-peroxidase complex, biotinyl tyramide (amplification reagent), and streptavidin peroxidase. The sections were visualized using diaminobenzidine and then counterstained with Mayer's haematoxylin.

Computer-assisted image analysis

The E-cadherin expression level was evaluated quantitatively using an image analysing system. The slides, after immunohistochemical staining, were examined using an Axiophot microscope (Carl Zeiss Co., Mainz, Germany) with a CCD camera, and then processed by an image analysing system (analySIS program; Image Analysis Co., Münster, Germany). Ten randomly selected images, containing the epithelial cells, were taken from each section with ×400 magnification. The input image was then transformed to a binary image that only contained two gray values (Fig. 1A, 1B). After processing to exclude the connective tissue area, the percentage of the immunostained surface from the epithelial tissue was calibrated.

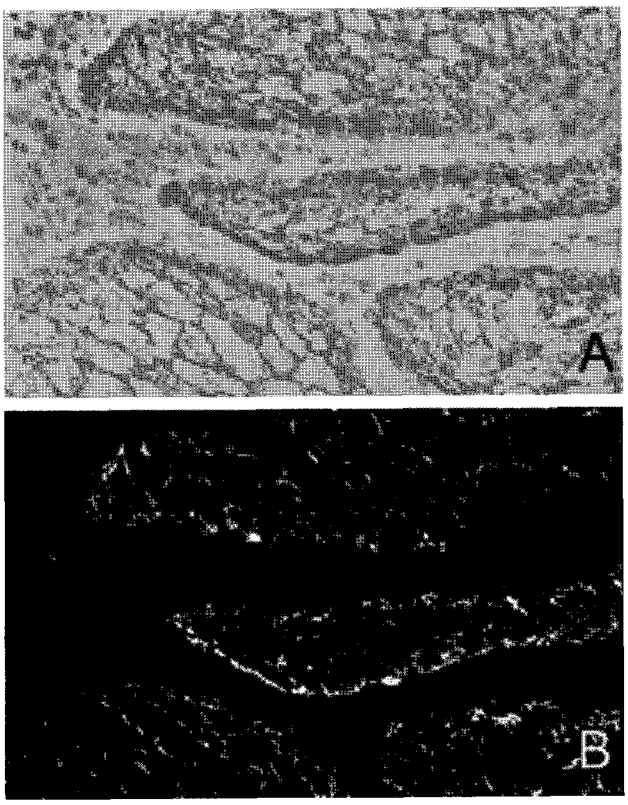

Fig. 1. Micrographs of a squamous cell carcinoma sample after E-cadherin immunostaining (A), and the corresponding binary image (B) treated using the colour extraction protocol. After processing to exclude the connective tissue area, the percentage of immunostained surface from the epithelial tissue was calibrated.

Extraction of DNA from the paraffin-embedded tissue

The samples were carefully microdissected prior to DNA isolation in order to avoid sample contamination with other cells. Using the H & E stained specimen as a template, 5-10 sections were microdissected with surgical scalpels by scraping the tissues into separate micro centrifuge tubes. After deparaffinization and hydration, the samples were lysed in 1 ml of 10 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 10 mM EDTA, 0.5% sodium dodecyl sulphate and 0.5 mg/ml proteinase K at 37°C for 24 hrs. The genomic DNA was then extracted using a QIAamp Tissue Kit (Cat. No. 29306, QIAGEN, CA) according to the manufacturer instructions. The quantity of extracted DNA was measured by a GeneQuant II (Pharmacia Biotech Inc., Cambridge, England) and diluted to concentration of 0.1 μg/μl.

Methylation-specific PCR

One microgram of the genomic DNA was denatured in 0.2 M NaOH for 10 min at 37°C. The sodium bisulfite treatment, which converts the unmethylated cytosine (but not the methylated cytosine) to uracil, was accomplished

using a CpGenome DNA Modification Kit (Intergen Co., Purchase, NY). The modified DNA was amplified using 2 pairs of primers (CpG Wiz™ E-cadherin Amplification Kit, Intergen Co.), which were designed to distinguish between the methylated and modified unmethylated sequences. Hot started PCR was performed using an automated thermal cycler (PTC-200, MJ research Inc., MA) with a final volume of 20 μl. The product sizes of the methylated and unmethylated reactions were 206 and 212 bp, respectively. Finally, 5 μl of each PCR reaction was loaded onto a 2% agarose gel and visualized under UV illumination. Prior to MSP, all the samples were amplified with a wild-type primer in order to confirm the quality of the extracted genomic DNA. The appropriate negative and positive controls were included in each PCR reaction. All the results were confirmed by duplicate analysis.

Statistical analysis

The correlations between the E-cadherin expression level and the clinicopathological parameters or the methylation status were analysed. Statistical analysis was performed using a Student *t*-test with SAS statistical software (version 6.12, SAS Institute Inc., Cary, SC). A P value < 0.05 was considered significant. The data for E-cadherin expression is presented as a mean ± SD (%).

Results

Both the benign oral epithelial lesions and SCCs showed positive staining with the HECD-1 monoclonal antibody, whereas the non-epithelial tissues such as submucosal connective tissues showed no staining. Most of the hyperplastic epithelium showed strong E-cadherin immunoreactivity at the cell-cell borders (Fig. 2A), whereas E-cadherin was variously expressed in the oral SCCs. There was a strong immunoreactivity in the well differentiated tumours and weak or negative immunoreactivity in moderately or poorly differentiated tumours (Fig. 2B,C).

Correlation of the E-cadherin expression level with the histopathological and clinical parameters

The mean E-cadherin expression level (% of stained cell surface/epithelial tissue), which was evaluated using an image analysing system, was 10.38% in the non-neoplastic epithelial hyperplasias and 5.50% in the squamous cell carcinomas, which showed a statistically significant difference ($P<0.05$) (Table 1). The results of E-cadherin immunostaining were correlated with histopathological differentiation. In the SCCs, the mean value of the immunostained surface was 6.75% and 3.73% in the well-differentiated group and the moderately or poorly differentiated group, respectively. The difference was statistically significant ($P<0.05$). However, there was no significant relationship between the E-cadherin expression level and the clinical stage.

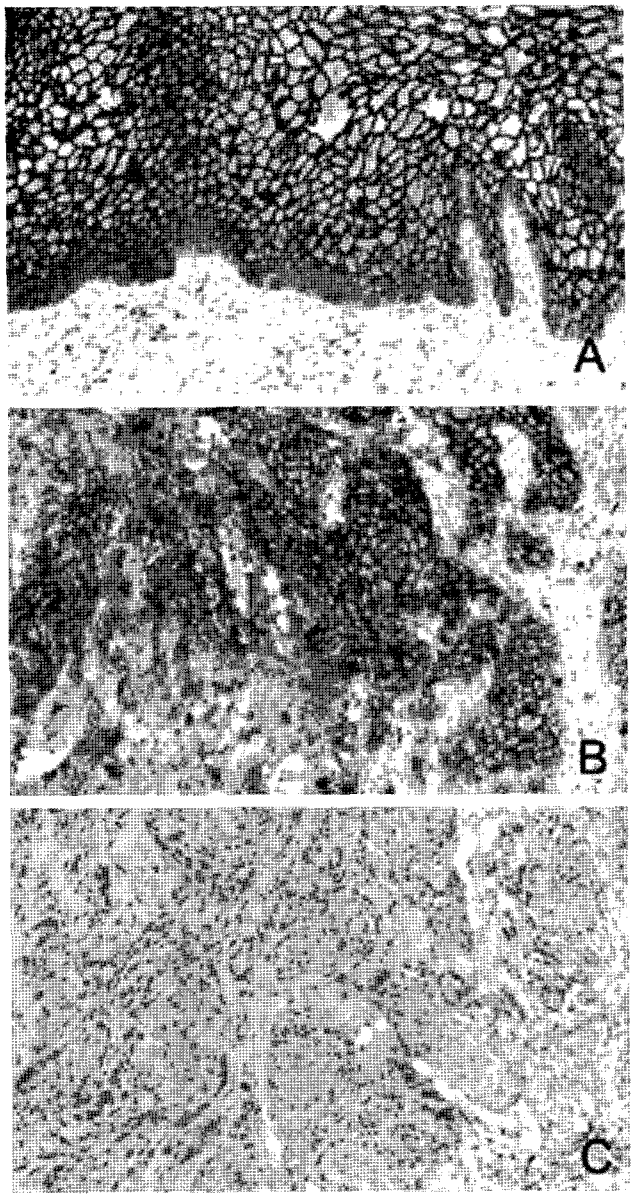

Figure 2. Immunohistochemical staining of E-cadherin using a CSA system. (A) Epithelial hyperplasia showing intensive immunoreactivity at the cell-cell borders of the epithelium. (B) Well differentiated SCC showing strong immunoreactivity between the tumour cells. (C) Moderately differentiated SCC showing a negative immunoreactivity in the tumour nests. Original magnification; 200 ×.

Correlation of E-cadherin expression with *CDH1* CpG methylation

The successful amplifications of the MSP were obtained from 8 out of 36 epithelial hyperplasias and 12 out of 33 oral SCCs. All the 8 amplified benign samples were unmethylated (Fig. 3A). In the oral SCCs, 5 out of 12 cases were methylated and the other 7 cases were unmethylated. In the five methylated tumours, four cases showed only a methylated (M) band and one case showed both methylated and unmethylated (U) bands (Fig. 3B). The mean values of

Table 1. E-Cadherin expression in relation to the clinicopathological characteristics and methylation status

Parameters Group	E-cadherin-positive area*	Statistics
Benign oral epithelial hyperplasia (n=33)	10.38 ± 5.25	$P < 0.05$**
Oral SCC (n=36)	5.50 ± 3.40	
Histopathologic differentiation (SCC)		
Well differentiated (n=21)	6.75 ± 3.36	$P < 0.05$**
Moderately or poorly differentiated (n=15)	3.73 ± 2.68	
Clinical stage (SCC)		
Stage I and II (n=10)	5.57 ± 2.26	$P > 0.05$
Stage III and VI (n=12)	5.96 ± 3.49	
Methylation status (SCC)		
Unmethylated (n=7)	7.28 ± 2.92	$P < 0.05$**
Methylated (n=5)	3.72 ± 1.46	

*Results expressed as mean ± standard error of the mean (%).
**Statistically significant
SCC, squamous cell carcinoma

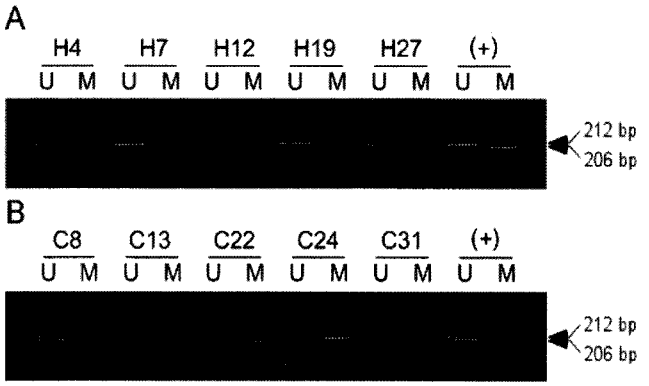

Fig. 3. Methylation specific PCR analysis of the *CDH1* promoter in epithelial hyperplasia (A) and oral SCC (B). Genomic DNA isolated from the paraffin-embedded tissue was modified by sodium bisulfite and analyzed by methylation specific PCR using two primers, one that amplifies the methylated DNA (M), and another that amplifies the unmethylated DNA (U). The amplified products were visualized by 2.0% agarose gel electrophoresis. The product sizes of the methylated and unmethylated reactions were 206 bp and 212 bp, respectively. Numbers at the top indicate the patient's identification.

the immunostained surface were 7.28% in the 7 unmethylated SCC samples and 3.72% in the methylated 5 samples (Table 1). Hypermethylation of the *CDH1* promoter was significantly associated with the reduced expression of the E-cadherin protein ($P<0.05$).

Discussion

One of the features of a malignancy is an invasion into the stroma and surrounding tissues. Cancer invasion is initiated by the dissociation of cells from the primary cancer nests, due to a loosened intercellular adhesion (Pittella *et al.*, 2001). The suppression of cell-cell adhesion might trigger the release of cancer cells from the primary cancer nests and confer invasive properties on a tumour (Chen *et al.*, 2003; Kajiyama *et al.*, 2003). The E-cadherin expression level is frequently reduced or absent in a variety of epithelial cancers, and a loss of normal intercellular junctions is believed to precede a tumour invasion and metastasis (Schipper *et al.*, 1991; Luo *et al.*, 1999; Saito *et al.*, 2004). The loss or reduction of E-cadherin-mediated adhesion is an important step in the development of an invasion and metastasis in many epithelial carcinomas, including head and neck carcinomas(Schipper *et al.*, 1991; Saito *et al.*, 2004). *In vitro* experiments showed that E-cadherin acts as an invasion-suppressor in human cancers (Luo *et al.*, 1999). However, the correlation between the E-cadherin expression level and the mechanism of a tumour cell invasion in oral cancer has not been fully elucidated.

This study immunohistochemically examined the E-cadherin expression level in a series of 36 oral SCCs and 33 oral epithelial hyperplasias. Most of the hyperplastic oral epithelium showed strong E-cadherin immunoreactivity at the cell-cell border, whereas heterogeneous expression was detected in the tumour cells. In oral SCCs, the mean value (% of stained cell surface/epithelial tissue) was 6.75 in the well-differentiated group and 3.73 in the moderately or poorly differentiated group. The E-cadherin expression level was significantly higher in the well differentiated tumours than in the moderately or poorly differentiated ones. This is in agreement with earlier studies showing that E-cadherin expression correlated with the differentiation status of the tumour (Schipper *et al.*, 1991; Chen *et al.*, 2003; Saito *et al.*, 2004). However, the extent of immunoreactivity could not be compared with previous studies because no quantitative immunohistochemical study of the E-cadherin expression level in an oral cancer has been reported. In most studies, the E-cadherin distribution in the cancer tissue was semi-

quantitatively evaluated and the tumours were classified into categories according to their E-cadherin expression patterns, such as preserved or strong and aberrant or weak expression (Chow *et al.*, 2001; Chang *et al.*, 2002).

There is increasing evidence that E-cadherin expression is associated with the differentiation of cancer, whereas contrasting results regarding the E-cadherin expression level and the patient prognosis have been reported. Some investigators have reported that E-cadherin expression is a significant prognostic factor for survival and recurrence (Chow *et al.*, 2001) and those tumours, which did not express E-cadherin, were more invasive than those showing E-cadherin expression (Schipper *et al.*, 1991; Chen *et al.*, 2003). Schipper *et al.* (1991) also reported that E-cadherin expression was inversely correlated both with the loss of differentiation in the tumour and with a lymph node metastasis in SCC of the head and neck. However, others reported no correlation between the E-cadherin expression level and the patient's prognosis (Andrews *et al.*, 1997). In this study, the clinical stage by the TNM system, which is a highly accepted predictive factor for a prognosis, did not show any correlation with the E-cadherin expression level. This is probably because of the low sample number or the inaccurate staging by a clinical examination only.

The transformation of normal epithelial cells into a carcinoma and the subsequent progression to an invasion and metastasis involve the accumulation of many genetic events, including mutations, a loss of heterozygosity (LOH) and transcriptional inactivation (Heppner and Miller, 1998). Recently, hypermethylation of the CpG islands around the 5' regulatory areas of the genes has attracted a great deal of attention as an alternative mechanism of the transcriptional inactivation of the tumour suppressor genes in human cancers (Hayashi *et al.*, 2001; Norrie *et al.*, 2003). CpG island methylation is associated with the changes in chromatin organization and the consequent repression of gene transcription. In normal tissues, CpG methylation is limited to exceptional situations, such as imprinted genes and inactive X chromosome (Li *et al.*, 1993). However, in a neoplasia, the aberrant methylation of CpG islands results in the inactivation of the tumour suppressor genes and the genes that maintain normal cell growth, such as *CDH1*, *p16*, *hMLH1* and *APC* (Chang *et al.*, 2002; Norrie *et al.*, 2003; Arai *et al.*, 2004; Esteller *et al.*, 2000). In *CDH1*, although a LOH and transcriptional defects can also affect the E-cadherin expression level, there is growing evidence that aberrant CpG methylation plays a substantial role in suppressing the transcription of this gene. In oral cancer, Saito *et al.* (1998) did not detect any mutation in the E-cadherin gene using a PCR-single strand conformation polymorphism. Gagnon *et al.* (2003) also showed that a treatment of the breast cancer cell lines lacking E-cadherin with a DNA methyltransferase inhibitor (5-deoxyazacytidine) elicits CpG island demethylation and the re-expression of E-cadherin.

In order to clarify the CpG methylation status of the *CDH1* promoter, the DNA samples were examined by MSP. All the amplified benign samples were unmethylated, whereas 42% of the oral SCCs were methylated. These results support that CpG methylation of E-cadherin may be involved in tumour progression. In our samples, one SCC showed both a methylated and unmethylated state. This might partly be a consequence of detecting some heterogeneity of the partial or hemi-methylation. It is also possible that normal infiltrating cells or contaminating stromal cells are responsible for some of the detected unmethylation. It is widely accepted that CpG methylation of the *CDH1* promoter causes a reduction in the E-cadherin expression level in a human cancer (Sarrio *et al.*, 2003; Topaloglu *et al.*, 2004). When the E-cadherin expression level was compared with the methylation status, the unmethylated tumours had a higher E-cadherin expression level than those with methylation.

In this study, prior to performing a MSP, the extracted genomic DNA was amplified using a wild-type primer (W) set, which could be annealed to any DNA (methylated or unmethylated). When the W primer cannot produce an amplification product (194 bp) with the genomic DNA, it is an indication of an inadequate quality of genomic DNA for a MSP. In this study, a relatively small number of samples were amplified. The efficiency of amplification appeared to be generally less than with the DNA extracted from the formalin fixed, paraffin-embedded tissue, even in a good yield, because the fixation procedure caused DNA degradation (Coates *et al.*, 1991). A further study using nested PCR will be needed to amplify the DNA extracted from the paraffin-embedded tissues.

In conclusion, *CDH1* methylation and the resultant reduction of E-cadherin expression might be associated with the progression of oral cancer. The correction of aberrant DNA methylation might provide a new strategy for cancer prevention and gene therapy by suppressing the epigenetic steps of oral carcinogenesis.

References

Andrews, N.A., Jones, A.S., Helliwell, T.R., and Kinsella, A.R.: Expression of the E-cadherin-catenin cell adhesion complex in primary squamous cell carcinomas of the head and neck and their nodal metastases. Br. J. Cancer **75**:1474-1480, 1997.

Arai, T., Esaki, Y., Sawabe, M., Honma, N., Nakamura, K., and Takubo, K.: Hypermethylation of the hMLH1 promoter with absent hMLH1 expression in medullary-type poorly differentiated colorectal adenocarcinoma in the elderly. Mod. Pathol. **17**:172-179, 2004.

Beahrs, O.H., Henson, D.E., Hutter, R.V.P., and Kennedy, B.J.: Manual for staging of cancer. 4th ed., pp 27-32, J.B. Lippincott, Philadelphia, 1992.

Broders, A.C.: Microscopic grading of cancer. Surg. Clin. North Am. **21**:974-961, 1941.

Chang, H.W., Chow, V., Lam, K.Y., Wei, W.I., and Yuen, A.:

Loss of E-cadherin expression resulting from promoter hypermethylation in oral tongue carcinoma and its prognostic significance. Cancer **94**:386-392, 2002.

Chen, H.C., Chu, R.Y., Hsu, P.N., Hsu, P.I., Lu, J.Y., Lai, K.H., Tseng, H.H., Chou, N.H., Huang, M.S., Tseng, C.J., and Hsiao, M.: Loss of E-cadherin expression correlates with poor differentiation and invasion into adjacent organs in gastric adenocarcinomas. Cancer Lett. **201**:97-106, 2003.

Chow, V., Yuen, A.P., Lam, K.Y., Tsao, G.S., Ho, W.K., and Wei, W.I.: A comparative study of the clinicopathological significance of E-cadherin and catenins (á,â,ã) expression in the surgical management of oral tongue carcinoma. J. Cancer Res. Clin. Oncol. **127**:59-63, 2001.

Coates, P.J., d'Ardenne, A.J., Khan, G., Kangro, H.O., and Slavin, G.: Simplified procedures for applying the polymerase chain reaction to routinely fixed paraffin wax sections. J. Clin. Pathol. **44**:115-118, 1991.

Esteller, M., Sparks, A., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M.A., Gonzalez, S., Tarafa, G., Sidransky, D., Meltzer, S.J., Baylin, S.B., and Herman, J.G.: Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. Cancer Res. **60**:4366-4371, 2000.

Gagnon, J., Shaker, S., Primeau, M., Hurtubise, A., and Momparler, R.L.: Interaction of 5-aza-2'-deoxycytidine and depsipeptide on antineoplastic activity and activation of 14-3-3sigma, E-cadherin and tissue inhibitor of metalloproteinase 3 expression in human breast carcinoma cells. Anticancer Drugs **14**:193-202, 2003.

Hayashi, K., Yokozaki, H., Goodison, S. Oue, N., Suzuki, T., Lotan, R., Yasui, W., and Tahara, E.: Inactivation of retinoic acid receptor beta by promoter CpG hypermethylation in gastric cancer. Differentiation **68**:13-21, 2001.

Heppner, G.H. and Miller, F.R.: The cellular basis of tumour progression. Int. Rev. Cytol. **177**:1-56, 1998.

Humar, B., Toro, T., Graziano, F., Muller, H., Dobbie, Z., Kwang-Yang, H., Eng, C., Hampel, H., Gilbert, D., Winship, I., Parry, S., Ward, R., Findlay, M., Christian, A., Tucker, M., Tucker, K., Merriman, T., and Guilford, P.: Novel germline CDH1 mutations in hereditary diffuse gastric cancer families. Hum. Mutat. **19**:518-525, 2002.

Kajiyama, H., Kikkawa, F., Khin, E., Shibata, K., Ino, K., and Mizutani, S.: Dipeptidyl peptidase IV overexpression induces up-regulation of E-cadherin and tissue inhibitors of matrix metalloproteinases, resulting in decreased invasive potential in ovarian carcinoma cells. Cancer Res. **63**:2278-2283, 2003.

Lei, H., Sjoberg-Margolin, S., Salahshor, S., Werelius, B., Jandakova, E., Hemminki, K., Lindblom, A., and Vorechovsky, I.: CDH1 mutations are present in both ductal and lobular breast cancer, but promoter allelic variants show no detectable breast cancer risk. Int. J. Cancer **98**:199-204, 2002.

Li, E., Beard, C. and Jaenisch, R.: Role for DNA methylation in genomic imprinting. Nature **366**:362-365, 1993.

Luo, J., Lubaroff, D.M., and Hendrix, M.J.: Suppression of prostate cancer invasive potential and matrix metalloproteinase activity by E-cadherin transfection. Cancer Res. **59**:3552-3556, 1999.

Norrie, M.W., Hawkins, N.J., Todd, A.V., Meagher, A.P., O'Connor, T.W., and Ward, R.L.: Inactivation of p16INK4a by CpG hypermethylation is not a frequent event in colorectal cancer. J. Surg. Oncol. **84**:143-150, 2003.

Pittella, F., Katsube, K., Takemura, T., Hashimoto. T., Kawano, T., Garrod, D., and Takagi, M.: Perinuclear and cytoplasmic distribution of desmoglein in esophageal squamous cell carcinomas. Pathol. Res. Pract. **197**:85-91, 2001.

Saito, T., Masuda, N., Miyazaki, T., Kanoh, K., Suzuki, H., Shimura, T., Asao, T., and Kuwano, H.: Expression of EphA2 and E-cadherin in colorectal cancer: correlation with cancer metastasis. Oncol. Rep. **11**:605-611, 2004.

Saito, Y., Takazawa, H., Uzawa, K., Tanzawa, H., and Sato, K.: Reduced expression of E-cadherin in oral squamous cell carcinoma: relationship with DNA methylation of 5' CpG island. Int. J. Oncol. **12**:293-298, 1998.

Sarrio, D., Moreno-Bueno, G., Hardisson, D., Sanchez-Estevez, C., Guo, M., Herman, J.G., Gamallo, C., Esteller, M., and Palacios, J.: Epigenetic and genetic alterations of APC and CDH1 genes in lobular breast cancer: relationships with abnormal E-cadherin and catenin expression and microsatellite instability. Int. J. Cancer **106**:208-215, 2003.

Schipper, J.H., Frixen, U.H., Behrens, J., Unger, A., and Birchmeier, W.: E-cadherin expression in squamous cell carcinomas of head and neck: inverse correlation with tumour dedifferentiation and lymph node metastasis. Cancer Res. **51**:6328-6337, 1991.

Sciubba, J.J.: Oral pathology: Clinical Pathologic Correlations, 4th ed., pp 52-72, Saunders, St. Louis, 2003.

Takeichi, M.: Cadherin cell adhesion receptors as a morphogenetic regulator. Annu. Rev. Cell Dev. Biol. **19**:207-235, 2003.

Topaloglu, O., Hoque, M.O., Tokumaru, Y., Lee, J., Ratovitski, E., Sidransky, D., and Moon, C.S.: Detection of promoter hypermethylation of multiple genes in the tumour and bronchoalveolar lavage of patients with lung cancer. Clin. Cancer Res. **10**:2284-2288, 2004.