

Expression of N-Cadherin in Salivary Gland Tumors

N. Kehagias^a A. Epivatianos^b L. Sakas^c D. Andreadis^b A. Markopoulos^b
K. Antoniadou^a

Departments of ^aOral and Maxillofacial Surgery and ^bOral Medicine and Oral Pathology, Dental School of Aristotle University of Thessaloniki, and ^cDepartment of Histopathology, 'G. Papanikolaou' General Hospital of Thessaloniki, Thessaloniki, Greece

Key Words

N-cadherin • Adhesion molecule • Salivary gland tumors • Immunohistochemistry • Epithelial-mesenchymal transition

Abstract

Objective: To detect immunohistochemically the N-cadherin expression in different types of benign and malignant salivary gland tumors in an attempt to note any possible correlation to their development, stage and invasive properties.

Materials and Methods: N-cadherin expression was examined in tissue specimens from 49 salivary gland tumors including: pleomorphic adenomas (4), Warthin's tumors (10), and myoepitheliomas (4) (benign tumors), as well as adenoid cystic carcinomas (14), mucoepidermoid carcinomas (4), polymorphous low-grade adenocarcinomas (6), and adenocarcinomas not otherwise specified (5) (malignant tumors). Twelve specimens of normal salivary glands were used as control. The perineural invasion and stage of malignant salivary gland tumors were evaluated. Immunohistochemical procedure was performed automatically using the Bond Polymer Refine Detection Kit. **Results:** N-cadherin expression was not found in normal salivary glands. In benign salivary gland tumors, N-cadherin along membranes of neoplastic cells as well as in centrocytes of lymphoid germinal centers was seen in 1 and 4 cases of Warthin's tumors, re-

spectively. Varied degree of N-cadherin expression was found in 13 (45%) cases of malignant salivary gland tumors. N-cadherin expression was significantly correlated with perineural invasion ($\chi^2 = 11.7$, $p < 0.0001$), but not with stage of malignant salivary gland tumors. **Conclusion:** N-cadherin expression was observed in malignant salivary gland tumors and could be an indicator of potentially aggressive behavior. N-cadherin expression by tumor cells could be attributed to perineural invasion.

Copyright © 2012 S. Karger AG, Basel

Introduction

Cadherins are a family of single-pass transmembrane glycoproteins which act as mediators in calcium-dependent cell-cell adhesion and play an essential role in development, cell polarity and tissues morphogenesis [1]. They are classified according to tissue patterns of expression: type E, epithelial-cell derived [2]; type P, placenta-derived [3], and type N, nerve-derived [4] are considered as 'classical cadherins'. Cadherins have a large extracellular domain for intercellular homotypic connections and a cytoplasmic domain which interacts with cytoplasmic proteins called catenins (α -, β - and γ -catenin) that link to the actin-based cytoskeleton [5, 6]. Catenins are critical

for signal transduction and various cellular functions [5, 7]. Normal epithelial cells typically express E-cadherin, whereas mesenchymal cells express N-cadherin [6].

N-cadherin was originally found in neural tissues [4], and later in smooth muscle cells [8], endothelial cells [9, 10], fibroblasts [11], myofibroblasts [12], and mast cells [13]. In cancer cells, expression of E-cadherin at cell-cell connections acts as an invasion suppressor complex [14], whereas N-cadherin in malignant cells of epithelial origin promotes cellular motility, migration, invasion and metastasis [15, 16]. N-cadherin has a dual role in the invasive process [17]. It promotes adhesive interactions among tumor cells with the microenvironment, and synergizes with the fibroblast growth factor receptor (FGFR) [18, 19], thereby inducing the invasiveness of tumor cells.

Studies concerning the expression of N-cadherin in normal salivary glands and their benign-malignant neoplasms are only rarely referred to in the literature focusing only on specific subtype of salivary gland malignancies [20].

The purpose of this study was to detect immunohistochemically the expression of N-cadherin in different types of benign and malignant salivary gland tumors in an attempt to note any possible correlation with their development, stage and invasive properties.

Materials and Methods

Forty-nine tissue specimens of benign and malignant salivary gland tumors, formalin-fixed, and paraffin-embedded were retrieved from the archives of Department of Histopathology of 'G. Papanikolaou' General Hospital and 'Theagenion' Anticancer Hospital, Thessaloniki, Greece. Benign salivary gland tumors included pleomorphic adenomas (n = 5), Warthin's tumors (n = 10), and myoepitheliomas (n = 4). Malignant salivary tumors included adenoid cystic carcinomas (ACC) (n = 14), mucoepidermoid carcinomas (MEC) (n = 4), polymorphous low-grade adenocarcinomas (PLGA) (n = 6), and adenocarcinomas not otherwise specified (NOS) (n = 5). The diagnosis of these cases was based on the established criteria of World Health Organisation (WHO) [21], and the stage of malignant tumors was obtained from patients' medical records. The tumors were subdivided into two groups: local tumors (stages I-II) and invasive/metastatic tumors (stages III-IV). New 3- μ m sections were cut and stained with hematoxylin and eosin and were reviewed for adequacy of tissue to study and agreement with the original diagnosis. Also, the sections from each specimen of malignant tumors were examined for the presence of perineural invasion. Twelve specimens of normal salivary tissue adjacent to benign neoplasms were used as control.

For the immunohistochemical detection of N-cadherin, antigen retrieval was performed including placement of the sections in a pressure cooker of 56°C for 24 h, following by microwave heat induced epitope retrieval for 20 min with Bond Epitope Retrieval

Solution 1 (AR 9961, Vision BioSystems, Newcastle Upon Tyne, UK). Endogenous peroxidase activity was quenched with 3% H₂O₂ in methanol for 10 min and then the sections were rinsed with Tris buffer (BDH Lab Suppliers, Poole, UK). The Bond Polymer Refine Detection Kit (DS9800, Vision BioSystems) was applied using the mouse primary monoclonal antibody N-cadherin (M-3612, Dako, Glostrup, Denmark) at a dilution 1:100 for 30 min. The procedure was performed using the autostainer Bond-max processing module (code 21005, Vision BioSystems) according to the manufacturer's instructions. Finally, the sections were washed in water and counterstained with hematoxylin. Sections incubated in Tris buffer and serum instead of the primary antibody were used as negative controls. The neural tissue of normal salivary glands was used as the internal positive control.

The localization, staining intensity and immunoreactivity were examined according to our previous study [22] as follows: the localization or staining pattern was classified as membranous and cytoplasmic when the immunostaining was localized in membrane and cytoplasm of cells, respectively. The staining intensity was assessed using the following brief evaluation: weak, moderate and strong. The sum of the staining intensity was used for the total immunoreactivity. The evaluation of immunoreactivity was performed as follows: 0 = negative or positive staining in \leq 2% of cells; \pm = positive staining in 3-10% of cells; + = positive staining in 11-50% of cells; ++ = positive staining in 51-70% of cells; +++ = positive staining in 70-90% of cells; ++++ = positive staining in 91-100% of cells. Five hundred cells from five fields on each section were enumerated as the percentage of cells expressing N-cadherin.

Perineural invasion and stage of malignant tumors in relation to N-cadherin expression were also evaluated. Sections were examined by two of the authors (A.E. and L.S.) independent of each other. Sections were re-examined when there was difference and agreement was reached by consensus.

For the statistical analysis the χ^2 test was used. Statistical significance was considered when p was <0.05 .

The whole study was performed according to the Declaration of Helsinki II and furthermore, the permission of the relevant hospitals was given for the review of the medical records.

Results

N-cadherin expression was not found in the epithelial cells of normal salivary glands. Immunoreactivity was seen in neural tissue, endothelial and smooth muscle cells of vessels and mast cells in the stroma. Also, in 5 cases focal immunostaining was observed along membranes of basal cells of normal oral squamous epithelium.

In the 20 benign salivary gland tumors, N-cadherin expression was not found in pleomorphic adenomas and myoepitheliomas. Unexpected immunoreactivity (++) was seen in membranes of epithelial cells in one case of Warthin's tumor (fig. 1a). In this case, careful re-examination of hematoxylin and eosin sections did not showed epithelial atypia, mitotic figures, necrosis or infiltration

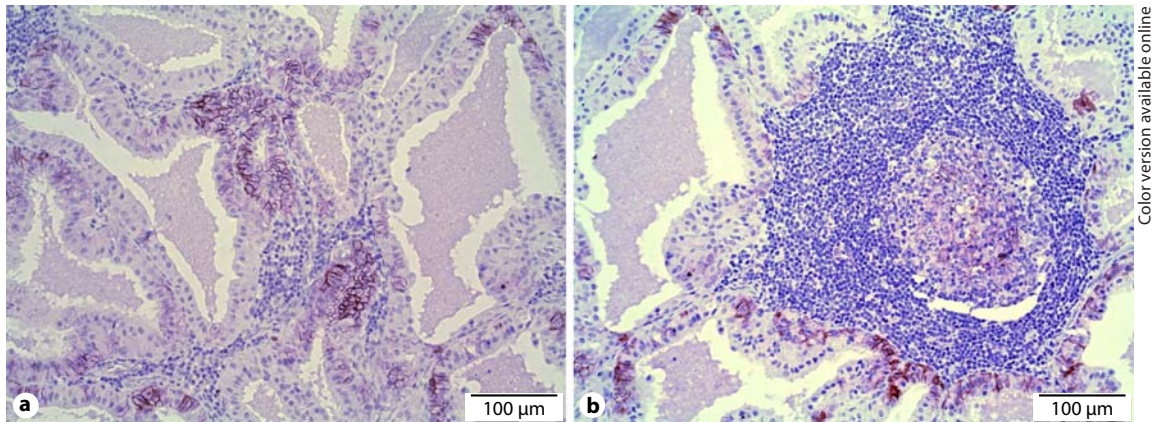


Fig. 1. a Warthin's tumor. Strong staining of N-cadherin in membranes of luminal and basal cells. **b** Moderate staining of N-cadherin in membranes of centrocytes of lymphoid tissue.

Table 1. Immunoreactivity and localization of N-cadherin in 13 positive cases of malignant salivary gland tumors

Type of tumor/ number of cases	Immunoreactivity/ number of cases	Localization	
		M	M/C
ACC/6	+: 1 ++: 1 +++: 4	6	
MEC/1	++: 1	1	
PLGA/2	+: 2	2	
ANOS/4	±: 1 +: 2 ++: 1	3	1

M = Membranous; C = cytoplasmic; ACC = adenoid cystic carcinoma; MEC = mucoepidermoid carcinoma; PLGA = polymorphous low-grade adenocarcinoma; ANOS = adenocarcinoma not otherwise specified.

around the tumor. Furthermore, according to the medical records, the 5-year follow-up of the patients did not show any recurrence or metastasis. Also expression of N-cadherin localized in membranes of centrocytes in germinal centers of lymphoid tissue was observed in 4 cases of Warthin's tumor (fig. 1b).

The 29 malignant salivary gland tumors showed N-cadherin expression in 13 (45%) cases. The immunoreactivity and localization of expression in these positive cases are presented in table 1. The immunoreactivity in the

Table 2. N-cadherin expression in relation to perineural invasion in 29 cases of malignant salivary gland tumors

Type of tumor/ number of cases	Cases without perineural invasion/N-cadherin expression	Cases with perineural invasion/N-cadherin expression
ACC/14	7/0	7/6
MEC/4	3/0	1/1
PLGA/6	4/1	2/1
ANOS/5	1/0	4/4
Total	15/1	14/12

ACC = Adenoid cystic carcinoma; MEC = mucoepidermoid carcinoma; PLGA = polymorphous low-grade adenocarcinoma; ANOS = adenocarcinoma not otherwise specified.

different types of malignant tumors was almost similar. The staining intensity in all positive cases varied from moderate to strong. Regarding the relation between N-cadherin expression and tumor stage our results showed positive expression in 11/24 of cases in stages I–II, and 2/5 of stages III–IV. N-cadherin expression was not significantly correlated with tumors' stage ($\chi^2 = 0.018$, $p < 0.2$). Perineural invasion in relation to N-cadherin expression is presented in table 2. N-cadherin expression was found in 1 case of the total 15 cases where perineural invasion was absent, and in 12 cases of the total 14 cases with perineural invasion. Perineural invasion was significantly correlated with N-cadherin expression ($\chi^2 = 11.7$, $p < 0.0001$).

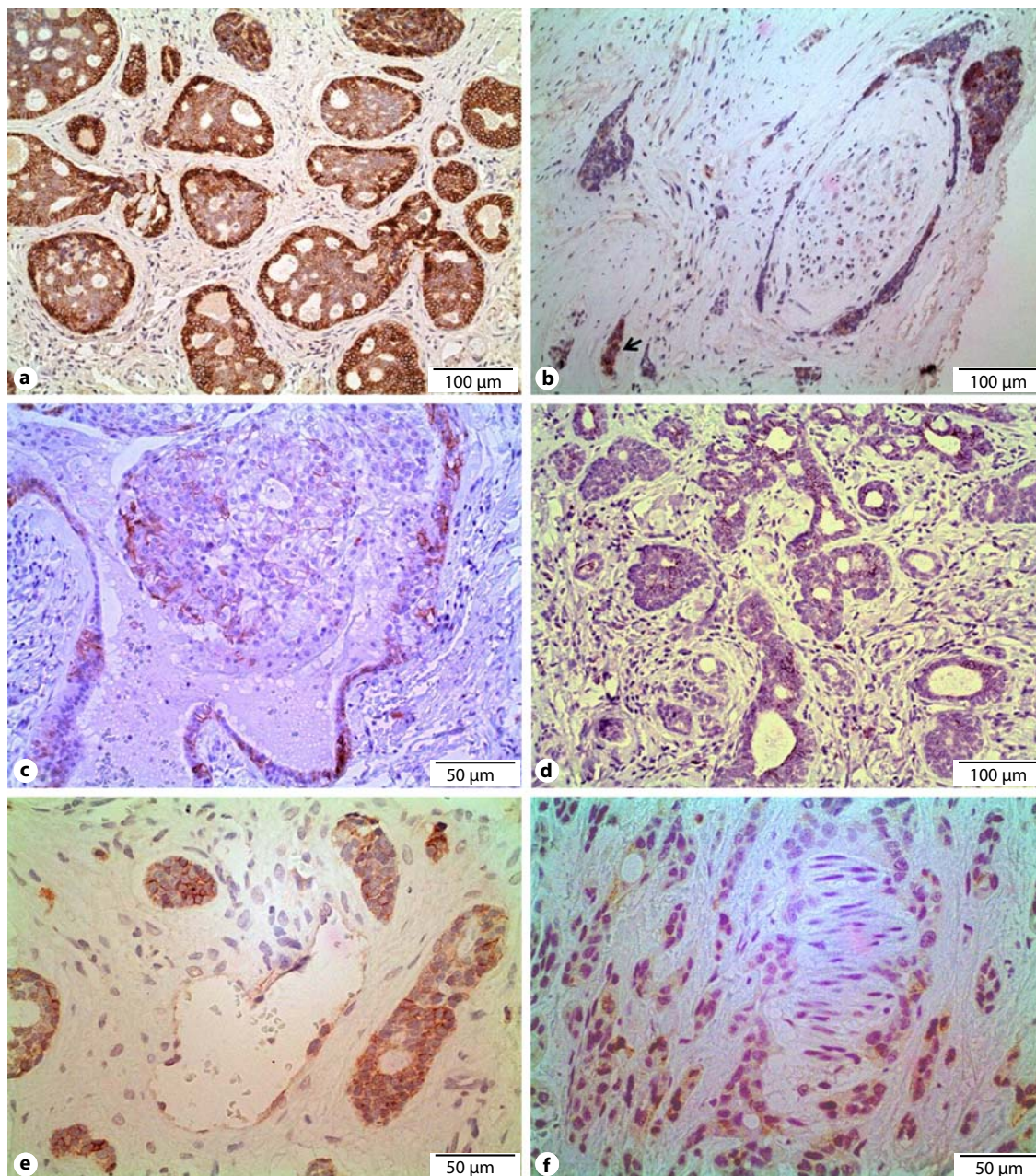


Fig. 2. N-cadherin in malignant salivary gland tumors. **a** Adenoid cystic carcinoma: strong expression along membranes of tumor cells in tubular, cribriform and solid histologic patterns. **b** Perineural and vascular invasion (arrow). Strong membranous staining of neoplastic cells. **c** Mucoepidermoid carcinoma: moderate N-cadherin staining on the surfaces of intercellular contacts. **d** Polymorphous low-grade adenocarcinoma: adenoid, tubular

and cribriform histologic patterns with strong N-cadherin staining in cell-cell contacts. **e** Adenocarcinoma not otherwise specified: moderate membranous positivity of tumor cells in solid and duct-like histologic patterns. Endothelial cells of vessel also express N-cadherin. **f** Perineural invasion, where N-cadherin staining is present in cell-cell contacts and cytoplasm of tumor cells.

In details, N-cadherin expression was observed in all histologic patterns (tubular, cribriform, solid) of ACCs (fig. 2a) and characteristically in tumor cells invading neural tissues and vessels (fig. 2b). All types of epithelial

cells in MECs were positively stained for N-cadherin (fig. 2c). The solid, cribriform, glandular, ductular, tubular and single-file histologic patterns were found to be positive for N-cadherin in PLGAs, but the presence and

proportions of these histologic patterns differed within the same case and among different cases (fig. 2d). Neoplastic cells in duct-like or glandular and solid patterns were also positive in all examined cases of NOS. Similar positivity was also observed in trabecular histologic pattern additionally presented in one case. N-cadherin expression was observed in all these histologic patterns of this tumor (fig. 2e, f).

Discussion

In the current study, N-cadherin was not found in the normal salivary gland epithelium. However, others [9, 10, 13, 20] had reported N-cadherin expression in membranes of basal cells in normal oral squamous epithelium, neural tissue, endothelial and smooth muscle cells of vessels, mast cells in the stroma and in the ductal cells of normal salivary glands. This difference could be explained by differences in immunohistochemical procedures [22] including the clonality of the primary antibody, the concentration of the primary and secondary antibodies, the length of incubation with such antibodies and the sensitivity of the enzymatic reaction [22].

In our study, N-cadherin expression was not observed in pleomorphic adenomas and myoepitheliomas but unexpectedly, N-cadherin expression was observed in membranes of epithelial cells in one case of Warthin's tumor. Epithelial cells normally express E-cadherin, as reported in our previous study [23]. The N-cadherin expression in this case of Warthin's tumor could be suggestive of a potential malignant transformation but careful re-examination of hematoxylin and eosin sections did not reveal epithelial atypia, mitotic figures, necrosis and infiltration of tissues adjacent to tumor. Furthermore, the five years that the patient was followed up did not show any recurrence or metastasis. Pathogenesis of Warthin's tumor has long been controversial with regards to the origin of both epithelium and lymphoid stroma. Although several theories have been proposed, the theory involving development from heterotopic glandular tissue in a lymph node continues to be the most substantiated pathogenesis [24]. It is possible that expression of N-cadherin in epithelial cells of Warthin's tumor to be transient during the development of heterotopic glandular tissue into lymphoid stroma with a process similar to that seen in gastrulation, where cells segregate from ectoderm, gradually cease to express E-cadherin and begin to express N-cadherin [25]. Noteworthy, in early stage of embryonic morphogenesis of mouse submandibular

gland, N- and E-cadherin display diffuse expression at cell-cell contacts, but at late stage of postnatal development, N-cadherin disappears while E-cadherin expression progressively increases [26]. Perhaps, N-cadherin expression in this case of Warthin's tumor indicates that the tumor is still in a developmental stage in contrast to the 9 cases with completed development. Further investigations will be necessary to confirm our finding and possibly explain the role of N-cadherin in Warthin's tumor. In many cases of Warthin's tumors, N-cadherin expression was observed, for the first time, in membranes of cells in germinal centers of lymphoid tissue. Centrocetes have the ability to migrate [27] and N-cadherin expression is possibly essential for this movement and contacts formation between them and follicular dendritic cells that originate from mesenchyme [28].

The immunohistochemical examination of twenty-nine malignant salivary gland tumors showed N-cadherin expression in 13 (45%) tumors. The N-cadherin expression was not significantly correlated with the stage of tumors including mucoepidermoid carcinoma as previously reported by Shieh et al. [20] who studied N-cadherin expression in mucoepidermoid carcinomas. However, they evaluated the presence of N-cadherin in mucoepidermoid carcinomas in comparison with the positive staining of N-cadherin in normal salivary gland tissue, as well. In contrast, our results revealed a *de novo* expression of N-cadherin in tumor cells in comparison to the absent expression of this molecule in the normal salivary gland tissues. This is probably due to a switching mechanism including the reduction of E-cadherin and the increase of N-cadherin expression during neoplastic process in salivary gland neoplasms as observed in several types of cancer [29, 30]. This reduced E-cadherin expression has been detected in cases of ACC, MEC, and PLGAs in our previous study [23].

E- to N-cadherin switching usually refers to the loss of E-cadherin expression and the gain of N-cadherin presence but may also include situations in which E-cadherin expression levels do not change significantly but the cells turn on a synchronous expression of N-cadherin, or other cadherins. Cells that express significant amounts of E-cadherin but only a small amount of N-cadherin may have increased motility [9].

In the present study, N-cadherin was almost totally absent in benign neoplasms, but was expressed almost in half of the malignant ones. This pattern of expression, together with the significant correlation between perineural invasion and N-cadherin expression in malignancies suggests a role for N-cadherin as an indicator

(biomarker) of potentially aggressive biologic behavior of malignant salivary gland tumors in terms of neoplastic cell motility and invasion enhancement especially via neural tissue. Interestingly, N-cadherin-bearing tumor cells gain motility, form transient contacts with the surrounding extracellular matrix, approach, interact and invade neural tissue [11] as well as vascular endothelium [15] that express N-cadherin also.

Conclusion

N-cadherin expression in malignant salivary gland tumors may be an indicator of potentially aggressive behavior. Perineural invasion likely is attributed to an N-cadherin expression by tumor cells.

Acknowledgement

Mr. N. Kehagias was supported financially by the Program 'Heraklitos II'.

References

- 1 Takeichi M: Cadherin cell adhesion receptors as a morphological regulation. *Science* 1991;251:1451-1455.
- 2 Takeichi M: Functional correlation between cell and cell adhesion properties and cell surface protein. *J Cell Biol* 1977;75:464-474.
- 3 Nose A, Takeichi M: A novel cadherin cell adhesion molecule: its expression patterns associated with implantation and organogenesis of mouse embryos. *J Cell Biol* 1986;103:2649-2658.
- 4 Hatta K, Okada TS, Takeichi M: A monoclonal antibody disrupting calcium-dependent cell-cell adhesion of brain tissues: possible role of its target antigen in animal pattern formation. *Proc Natl Acad Sci USA* 1985;82:2789-2793.
- 5 Ozawa M, Ringwald M, Kemler R: Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. *Proc Natl Acad Sci USA* 1990;87:4246-4250.
- 6 Wheelock M, Knudsen K, Johnson K: Membrane-cytoskeleton interactions with cadherin cell adhesion proteins: role of catenins as linker proteins. *Curr Top Membr* 1996;43:169-185.
- 7 Nagafuchi A, Takeichi M: Cell binding function of E-cadherin is regulated by the cytoplasmic domain. *EMBO J* 1988;7:3679-3684.
- 8 Molseeva EP: Adhesion receptors of vascular smooth muscle cells and their functions. *Cardiovasc Res* 2001;52:372-386.
- 9 Wheelock M, Shintani Y, Maeda M, Fukumoto Y, Johnson K: Cadherin switching. *J Cell Sci* 2008;121:727-735.
- 10 Mariotti A, Perotti A, Sessa C, Ruegg C: N-cadherin as a therapeutic target in cancer. *Expert Opin Investig Drugs* 2007;16:451-465.
- 11 Matsuyoshi N, Imamura S: Multiple cadherins and expression in human fibroblasts. *Bioch Biophys Res Com* 1997;235:355-358.
- 12 De Wever O, Mareel M: Role of tissue stroma in cancer invasion. *J Pathol* 2003;200:429-447.
- 13 Suzuki A, Suzuki R, Furuno T, Teshima R, Nakanishi M: N-cadherin plays a role in the synapse-like structures between mast cells and neuritis. *Biol Pharm Bull* 2004;27:1891-1894.
- 14 Behrens J, Mareel M, Van Roy F, Birchmeier W: Dissecting tumor cell invasion: epithelial cells acquire invasive properties following the loss of uvomorulin-mediated cell-cell adhesion. *J Cell Biol* 1989;108:2435-2447.
- 15 Islam S, Carey T, Wolf G, Wheelock M, Johnson K: Expression of N-cadherin by human squamous carcinoma cells induces a scattered fibroblastic phenotype with disrupted cell-cell adhesion. *J Cell Biol* 1996;135:1643-1654.
- 16 Hazan R, Phillips G, Qiao R, Norton I, Aaronson S: Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. *J Cell Biol* 2000;148:779-790.
- 17 Nagi C, Guttman M, Jaffer S, Qiao R, Keren R, Triana A, Li M, Godbold J, Bleiweiss I, Hazan R: N-cadherin expression in breast cancer: correlation with an aggressive histologic variant-invasive micropapillary carcinoma. *Breast Cancer Res Treat* 2005;94:225-235.
- 18 Nieman M, Prudoff R, Johnson K, Wheelock M: N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. *J Cell Biol* 1999;147:631-644.
- 19 Suyama K, Shapiro I, Guttman M, Hazan R: A signaling pathway leading to metastasis is controlled by N-cadherin and the FGF receptor. *Cancer Cell* 2002;2:301-314.
- 20 Shieh Y, Chang L, Chin K, Wu C, Lee H: Cadherin and catenin expression in mucopidermoid carcinoma: correlation with histopathologic grade, clinical stage, and patient outcome. *J Oral Pathol Med* 2003;32:297-304.
- 21 WHO Histological Classifications of Tumours of the Salivary Glands: In Barnes L, Eveson J, Reichart P, Sidransky D (eds): *World Health Organization Classification of Tumours. Pathology and Genetics of Head and Neck Tumours*. Lyon, IARC Press, 2005, pp 209-281.
- 22 Epivatianos A, Iordanidis S, Zaraboukas T, Antoniadis D: Adenoid cystic carcinoma and polymorphous low-grade adenocarcinoma of minor salivary glands: a comparative immunohistochemical study using the epithelial membrane and carcinoembryonic antigen. *Oral Dis* 2005;11:175-180.
- 23 Andreadis D, Epivatianos A, Mireas G, Nomikos A, Pouloupoulos A, Yiotakis J, Barbatis C: Immunohistochemical detection of E-cadherin in certain types of salivary gland tumours. *J Laryngol Otol* 2006;120:298-304.
- 24 Warnock G: Papillary cystadenoma lymphomatosum (Wartin's tumor); in Ellis G, Auclair P, Gnepp D (eds): *Surgical Pathology of the Salivary Glands*. Philadelphia, Saunders, 2001, pp 187-201.
- 25 Takeichi M: Cadherins: a molecular family essential for selective cell-cell adhesion and animal morphogenesis. *Trends Genet* 1987;3:213-217.
- 26 Menko A, Zhang L, Kreidberg J, Kukuruzinska M: Regulation of cadherin junctions during mouse submandibular gland development. *Dev Dyn* 2002;224:321-333.
- 27 Beltman J, Allen C, Cyster J, de Boer R: B cells within germinal centers migrate preferentially from dark to light zone. *Proc Natl Acad Sci USA* 2011;108:8755-8760.
- 28 Vinuesa C, Linterman M, Goodnow C, Rantal K: T cells and follicular cells in germinal center. B-cell formation and selection. *Immunol Rev* 2010;237:72-89.
- 29 Gravdal K, Halvorsen O, Haukaas S, Akslen I: A switch from E-cadherin to N-cadherin expression indicates epithelial to mesenchymal transition and is of strong and independent importance for the progress of prostate cancer. *Clin Cancer Res* 2007;13:7003-7011.
- 30 Han R, Soler A, Knudsen K, Salazar H: Distinct cadherin profiles in special variant carcinomas and other tumors of the breast. *Hum Pathol* 1999;30:1035-1039.