TUNEL as a Tumor Marker of Tongue Cancer

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Abstract. *Squamous cell carcinoma (SCC) of the tongue has an increasing incidence, a high morbidity rate and a 50% 5-year mortality rate. We analyzed the rate of apoptosis using TDT-mediated dUTP-biotin nick end-labeling (TUNEL), p53 and heparanase in 73 patients with tongue cancer by immonohistochemistry, and tested data for correlation with survival, tumor size, grade and metastasis. TUNEL staining was strong in 54% of the tumors; the remainder lacked staining, as did all healthy control tissues. Significant correlations were found between TUNEL staining level and p53 expression rates (p=0.016) and between TUNEL and heparanase (p=0.039). Moreover, while the cumulative 5-year survival probability for tumors not stained for TUNEL and p53 was 63%, but dropped to 34% with TUNEL staining; for lack of TUNEL and heparanase (=0), 5-year survival rate was 50%, while staining presence (>0) reduced survival to 34%. TUNEL joins other biomarkers in indicating prognosis of understanding pathogenesis of tongue cancer.*

Squamous cell carcinoma (SCC) of the tongue is a common malignancy of the oral cavity with an increasing incidence (especially among young people), and a 5-year mortality rate of approximately 50% (1-3). This poor survival rate has not changed significantly in the past half-century despite the progress made in the fields of surgery, radiotherapy and adjuvant chemotherapy, and in imaging and diagnosis (4-7). Moreover, patients who survive often suffer extreme unpleasantness as the treatment frequently results in significant mutilation of the tongue and oral cavity, and compromised speech, taste, mastication, and swallowing. (8). Further improvement of the survival rate may be arise from a better understanding of the biological nature of the disease which, in turn, may facilitate the development of more efficient tools for the detection and treatment of this form of cancer. Although the prognosis and the therapeutic modality offered to patients are often related to the stage and histological grading of the tumor, these are subjective and unreliable predictors. Identification of biological factors indicative of the aggressiveness of the specific tumor is desirable, especially as the biological characteristics of these tumors are variable even when their staging is identical.

In the current study, immunohistological examination of the following was performed: the terminal deoxynucleotidyl transferase (TdT) mediated biotinylated deoxyuridine-triphosphate (dUTP) nick end-labeling (TUNEL) (for apoptosis rate assessment), the carcinogenesis- *vs*. apoptosis-related marker p53 and the metastasis–angiogenesis-related marker heparanase. The analysis of these markers was made because they represent levels of tumor carcinogenesis, apoptosis and aggressiveness. The percentage of apoptotic cells labeled by the well-established TUNEL method, which enables the visualization of apoptotic cells using an *in situ* end-labeling technique that labels DNA breaks in apoptotic cells (9). Mutation in the *p53* gene which results in encoded non-functional protein is considered to be the most common genetic event in human cancer. It has been suggested that mutated p53 may lead to carcinogenesis, as the wild-type p53 contributes to tumor suppression through at least two mechanisms in response to DNA damage, namely arrest of cell proliferation and induction of apoptosis (9-13).

The purpose of the current study was to analyze all three markers in a relatively large cohort of patients and to test the data obtained for correlation with the survival rates, as well as with other specified clinical tools currently available for prognosis prediction.

Materials and Methods

Patients and experimental design. A total of 73 patients with a mean age of 61±12 years (range of 15-90 years) and a gender distribution of 35 (48%) males and 38 (52%) females, all of whom were diagnosed with tongue cancer, were enroled in the current study.

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Data concerning clinical tumor characteristics were collected and analyzed: grade of tumor, size (T), neck metastasis (N), distant metastasis (M) and the existence/absence of extracapsular spread of these neck metastases.

Pathological study. All specimens had been formalin-fixed and paraffin-embedded following surgical harvesting. Shortly prior to the immunological evaluation, serial sections (4 μm in thickness) were prepared for hematoxylin and eosin staining, p53 and heparanase immunostaining and for nick end-labeling (TUNEL) staining.

TUNEL staining. Sections were stained by the *in situ* Death Detection POD kit (Roche Diagnostic GmbH, Mannheim, Germany), according to the manufacturer's instructions. Briefly, after deparaffinization and rehydration, sections were incubated with proteinase K (20 μm/ml in 10 mM Tris/HCl pH 7.4) for 30 min at 37˚C. Slides were rinsed with phosphate-buffered saline (PBS) and incubated with 3% H_2O_2 in methanol for 10 min at room temperature to block endogenous peroxidase activity, followed by PBS washing and incubation in 0.1% Triton® X-100 in 0.1% sodium citrate for 2 min on ice (4˚C). Sections were incubated with a mixture of TdT solution and fluorescein isothiocyanate dUTP solution in a humidified chamber at 37˚C for 60 min. This was followed by washings with PBS and incubation with anti-fluorescein antibody Fab fragments conjugated with horse-radish peroxidase in a humidified chamber at 37˚C for 30 min. After washing with PBS, amino ethyl carbazole (AEC) solution was applied, followed by light counterstain with hematoxylin. Paraffin-embedded sections of normal tonsils were used as positive control. Negative control was obtained by replacing the TdT solution with distilled water. The presence of clear positive red-brown nuclear staining (AEC chromogen) was indicative of apoptotic cells. At least 1,000 tumor cell nuclei were examined in the most evenly and distinctly labeled areas. The number of TUNEL-positive tumor cell nuclei was counted and the apoptotic index was the percentage of apoptotic cells in the tumor. The apoptotic indices were classified into three groups: less than 1% (0), 1-3% (1) and over 3% (2).

P53 staining. Four-micrometer paraffin-embedded sections were dewaxed and rehydrated. Endogenous peroxidase was blocked by incubation with 3% H_2O_2 in methanol for 10 min. Nonspecific binding was blocked by incubation in 10% normal serum for 20 min. Sections were heated in a microwave oven at 800 W for 15 min in 10 mM citrate buffer (pH 6). The antibodies used in the p53 study were anti-p53 (clone BP 53.12; 1:100; Zymed Laboratories, San Francisco CA, USA).

Slides were incubated with the antibodies and antiserum for 60 min at room temperature, followed by the application of the streptavidin-biotin complex method (Histostain Plus, Zymed Laboratories). Color development was performed with AEC followed by light hematoxylin counterstaining. Positive controls of colonic tumor, known to show strong p53 expression, were run in parallel. At least 500 tumor cells were counted in the areas with strongest staining. Staining for p53 was considered positive when more than 10% of tumor cells showed strong nuclear staining: Staining of 10-30% of cells was classified as +1; staining of over 30% of cells was classified as +2.

Heparanase immunostaining. Staining of formalin-fixed, paraffin-embedded 5 μm sections for heparanase was performed. Briefly, slides were deparaffinized, rehydrated and endogenous peroxidase activity was quenched (30 min) by 3% hydrogen peroxide in methanol. Slides were then subjected to antigen retrieval by boiling (20 min) in 10 mM citrate buffer (pH 6). Slides were incubated with 10% normal goat serum (NGS) in PBS for 60 min to block nonspecific binding and incubated for 20 h at 4˚C with anti-heparanase 733 antibody diluted 1:100 in blocking solution. Antibody 733 was raised in rabbits against a 15 amino acid peptide (KKFKNSTYSRSSVDC) that maps to the *N*-terminus of the 50 kDa heparanase subunit and preferentially recognizes the 50 kDa heparanase subunit *vs.* the 65 kDa latent pro-enzyme (Zestler). Slides were extensively washed with PBS containing 0.01% Triton® X-100 and incubated with a secondary reagent (Envision kit) according to the manufacturer's (Dako, Glostrup, Denmark) instructions. Following additional washes, color was developed with the AEC reagent (Dako), sections were counterstained with hematoxylin and mounted, as described. Immunostained specimens were examined by a senior pathologist (OBI) who was blind to the clinical data of the patients and were scored according to the intensity of staining (0: none, 1: weak; 2: strong). Specimens that were similarly stained with pre-immune serum or applying the above procedure but lacking the primary antibody, yielded no detectable staining. In all tumors diagnosed as heparanase-positive, more than 50% of the cells reacted with the anti-heparanase antibody.

Statistical evaluation. Data concerning demographics, markers and other clinical characteristics of the patients were calculated and analyzed. For categorical variables, frequencies and relative frequencies were calculated. For continuous variables, ranges, medians, means and standard errors were computed. The Pearson correlation was used to analyze the correlation between patient characteristics. The Chi-square test of independence was used to compare the relative frequencies of the categorical variables. For small numbers of observations (at least one cell with less than five observations), the Fisher-Irwin exact test was used. The Kaplan-Meier method was used to calculate the probability of survival as a function of time among all patients and in subgroups of patients (by stage, grade, spreading and locality). The log-rank test was used to compare between pairs of Kaplan-Meier curves.

Table I. *TUNEL staining levels of tumors from tongue cancer patients.*

TUNEL level	No. patients (%)
0	34 (46)
1	34 (46)
2	5 (8)
Total	73 (100)

Results

TUNEL and clinical data. A lack of TUNEL expression (staining level =0) was found in 34 patients (46%), moderate staining (=1) was found in another 34 patients (46%), and in the remaining 5 (8%) TUNEL staining was strong (=2) (Table I). No significant correlation between the TUNEL staining level and the grade, T, N, M or extracapsular spread

Table II. *TUNEL staining level as related to histopathological grading.*

Tumor grade	0 No. patients (%)	>0 No. patients (%)
1	18 (53)	16 (41)
2	13 (38)	13 (33)
3	2 (6)	8 (21)
Not determined	1 (3)	2 (5)
Total	34 (100)	39 (100)

Table III. *TUNEL attaining level as related to the clinical indictors: T (a), N (b) and M (c).*

a.

T	0 No. patients (%)	>0 No. patients (%)
1	12 (35)	9 (23)
2	16 (47)	21 (54)
3	3 (9)	3 (8)
4	2 (6)	2 (5)
Not determined	1 (3)	4 (10)
Total	34 (100)	39 (100)

b.

N	0 No. patients (%)	>0 No. patients (%)
0	23 (68)	25 (64)
1	4 (12)	4 (10)
2	6 (17)	7 (18)
Not determined	1 (3)	3 (8)
Total	34 (100)	39 (100)

c.

M	0 No. patients (%)	>0 No. patients (%)
0	32 (94)	36 (92)
Not determined	2 (6)	3 (8)
Total	34 (100)	39 (100)

was noted (Tables II and III). In 44 (60%) patients, the SCC lesion arose without known etiology (*de novo*) while in 12 (16%) patients there was a pre-malignant lesion such as leukoplakia, erythroleukoplakia or lichen planus which had been biopsied and monitored for up to 10 years prior to the definitive diagnosis of SCC. Twenty-five patients (24%) smoked and/or drank alcohol prior to the SCC diagnosis. No significant differences were found among the levels of TUNEL staining in these three subgroups (smokers, *de novo* or pre-malignant).

Table IV. *TUNEL staining level as related to staining level of p53 (a) and heparanase (b).*

a.

p53	0 No. patients (%)	>0 No. patients (%)
0	16 (47)	7 (18)
1		5 (13)
2	8 (24)	7 (18)
3	8 (24)	14 (36)
Not determined	2 (5)	6 (15)
		(p=0.016)

b.

Heparanase	0 No. patients (%)	>0 No. patients (%)
0 [0-0.5]	17 (50)	13 (33)
1 [1-1.5]	11 (32)	7 (18)
2 [2-2.5]	3 (9)	6 (15)
Not determined	3 (9)	13 (33)
		(p=0.039)
Total	34 (100)	39 (100)

TUNEL, p53 and heparanase immunostaining and survival. As can be seen in Table IV, significant correlations were found between the TUNEL staining level and that of both p53 (p=0.016) and heparanase (p=0.039). As can be seen in Figure 1, the TUNEL staining did not alter the survival probability of the patients. However, upon examination of the cases that were negative for p53 (=0), there was a clear trend for reduced survival in patients whose TUNEL staining level was positive (cumulative survival rates at 5 years dropped from 63% to 34%), though this did not reach statistical significance (Figure 2). Similarly, the cumulative 5-year survival rate of the TUNEL=0, heparanase=0 group was 50%, but 34% in the TUNEL>0, heparanase>0 group (Figure 3).

Discussion

TUNEL immunostaining was found to be positive (>0) in most of the tongue cancer specimens analyzed (in 54% of the cases) as compared with healthy controls (in which it was

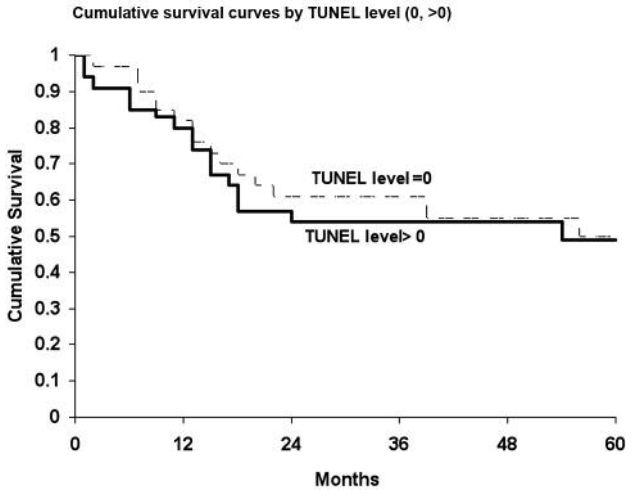

Figure 1. *Survival probability curves of tongue cancer patients according to TUNEL immunostaining levels. Kaplan-Meier analysis (age-adjusted) showed similar survival rates (50% at 5 years) in patients with either positive or negative TUNEL expression.*

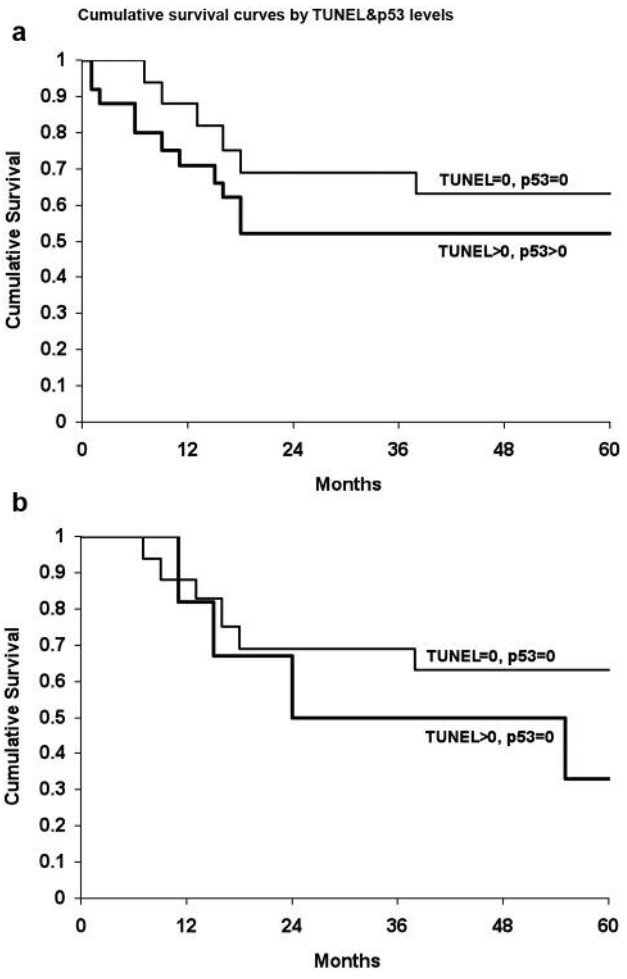

Figure 2. *Survival probability curves of tongue cancer patients according to TUNEL and p53 immunostaining levels. Kaplan-Meier analysis (age-adjusted) showed lower (though not statistically different) survival rates in patients with tumors positive for TUNEL and p53 (a) and in those of the p53-negative group who were TUNEL-positive (b).*

always negative=0), suggesting TUNEL as a possible indicator in the carcinogenic process. This finding is in accord with previous studies (14, 15). Furthermore, TUNEL staining significantly correlated with those of both p53 and heparanase, also indicating the possible role of TUNEL, p53 and heparanase collectively in tongue carcinogenesis. Most interestingly, the presence of TUNEL, together with p53 and heparanase seems to reduce survival rates of the patients - while TUNEL presence alone did not reduce survival, the combination of TUNEL presence and positive p53 reduced survival rate substantially. Similarly, in the group of patients where p53 was negative, those with TUNEL presence were found to have lower survival rates (Table II). The fact that TUNEL by itself did not demonstrate a significant effect is supported by the correlation analysis which did not show significant correlation between TUNEL staining and grade, T, N, or M scores.

The fact that its presence may indicate a mutual carcinogenetic role with either heparanase or p53 is not surprising, as p53 is considered to be an established marker of carcinogenesis and proliferation (in spite of the fact that our currently-employed immunostaining analysis of p53 may not fully reflect the mutational status of this gene). Moreover, traditionally, heparanase activity has been implicated in cellular invasion associated with cancer metastasis (16-18). This notion recently gained further support by employing siRNA and ribosome technologies, clearly depicting heparanase-mediated heparan sulfate cleavage and extracellular matrix remodeling as critical requisites for angiogenesis and metastatic spread (19). More recently, heparanase up-regulation was documented in an increasing number of primary human tumors. Similarly, heparanase up-regulation correlated with increased lymph node and distant metastasis (20-22), collectively providing strong clinical support for the pro-metastatic feature of the enzyme. In any case, the exact biological meaning of strong TUNEL staining in tongue cancer is yet to be elucidated. TUNEL *in situ* technique for the detection of apoptosis is not completely specific, as overlap between apoptotic and necrotic cell death has been reported, which may result in the fact that some of the apoptotic cells do not stain (23, 24). TUNEL evaluation and scoring is a difficult task for clinical application. Early DNA fragmentation may not be readily detected by the TUNEL assay and this may lead to underestimation of the apoptotic values. Moreover, the margin of error in scoring TUNEL between cases is too narrow to allow for definitive

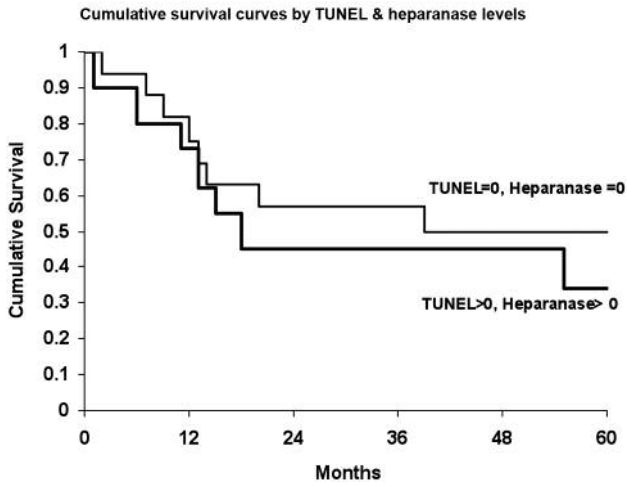

Figure 3. *Survival probability curves of tongue cancer patients according to TUNEL and heparanase immunostaining levels. Kaplan-Meier analysis (age-adjusted) showed lower (though not statistically different) survival rates in patients with positive TUNEL and heparanase expression.*

categorization. The report of Grast-Kraupp *et al*. (25), which found that TUNEL failed to distinguish between apoptotic and necrotic cells, strengthens this point.

Thus, the exact biological pathway expressed by TUNEL staining is yet to be fully elucidated, and its exploration is highly warranted, in light of the fact that most tongue cancer tumors exhibit moderate and strong levels of TUNEL staining, which is a pathognononic and a reproducible finding.

Moreover, the demonstrated concurrent presence of TUNEL staining in tongue cancer tumors along with p53 and heparanase indicates a mutual role for these various biological pathways involved in the pathogenesis of tongue cancer. Further analysis of the role of these markers in the pathogenesis of tongue cancer is warranted. This may lead to finding a therapeutic cellular target for this lethal cancer which may or may not be associated with p53 and/or heparanase.

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References

1 Kantola S, Parikka M, Jokinen K, Hyrynkangs K, Soini Y, Alho OP and Salo T: Prognostic factors in tongue cancer – relative importance of demographic, clinical and histopathological factors. Br J Cancer *83*: 614-619, 2000.

2 Myers JN, Elkins T, Roberts D and Byers RM: Squamous cell carcinoma of the tongue in young adults: increasing incidence and factors that predict treatment outcomes. Otolaryngol Head Neck Surg *122*: 44-51, 2000.

3 Bensinger W, Schubert M, Ang KK, Brizel D, Brown E, Eilers JG, Elting L, Mittal BB, Schattner MA, Spielberger R, Treister NS and Trotti AM 3rd. NCCN task force report: Prevention and management of mucositis in cancer care. J Natl Compr Canc Netw *6(Suppl 1)*: S1-21, 2008.

4 Nagler RM, Braun Y, Daitzchman M and Laufer D: Spiral CT angiography – an alternative vascular evaluation technique for head and neck microvascular reconstraction: a preliminary experience. Plast Reconst Surg *100*: 1697-1703, 1997.

5 Ribeiro KC, Kowalski LP and Latorre MR: Impact of comorbidity, symptoms, and patients' characteristics on the prognosis of oral carcinomas. Arch Otolaryngol Head Neck Surg *126*: 1079-1085, 2000.

6 Silverman S Jr: Mucosal lesions in older adults. J Am Dent Assoc *139*: 252-253, 2008.

7 Muir C and Weiland L: Upper aerodigestive tract cancers. Cancer *75*: 147-153, 1995.

8 Montes DM and Schmidt BL. Oral maxillary squamous cell carcinoma: Management of the clinically negative neck. J Oral Maxillofac Surg *66*: 762-766, 2008.

9 Campo-Trapero J, Cano-Sanchez J, Palacios-Sanchez B, Sanchez-Gutierrez JJ, Gonzalez-Moles MA and Bascones-Martinez A: Update on molecular pathology in oral cancer and precancer. Anticancer Res *28*: 1197-1205, 2008.

10 Attardi LD, Lowe SW, Brugarolas J and Jacks T: Transcriptional activation by p53, but not induction of the *p21* gene, is essential for oncogene-mediated apoptosis. EMBO J *15*: 3693-3701, 1996.

11 Xie X, Clausen OPF, De Angelis P and Boysen M: The prognostic value of spontaneous apoptosis, Bax, Bcl-2, and p53 in oral squamous cell carcinoma of the tongue. Cancer *86*: 913-920, 1999.

12 D'Silva NJ and Ward BB: Tissue biomarkers for diagnosis and management of oral squamous cell carcinoma. Alpha Omegan *100*: 182-189, 2007.

13 Nagler RM, Barak M, Ben-Aryeh H, Peled M, Filatov M and Laufer D: Early diagnostic and treatment monitoring role of Cyfra, 21-1 and TPS in oral squamous cell carcinoma. Cancer *35*: 1018-1025, 1999.

14 Loro LL, Vintermyr OK, Liavaag PG, Jonsson R and Johannessen AC: Oral squamous cell carcinoma is associated with decreased Bcl-2/Bax expression ratio and increased apoptosis. Hum Pathol *30*: 1097-1105, 1999.

15 Nagler RM, Kerner H, Ben-Eliezer S, Minkov I and Ben-Itzhak O: Prognostic role of apoptopic, Bcl-2, c-erbB-2 and p53 tumor markers in salivary gland malignancies. Oncology *64(4)*: 389-398, 2003.

16 Vlodavsky I, Fuks Z, Bar-Ner M, Ariav Y and Schirrmacher V: Lymphoma cells mediated degradation of sulfated proteoglycans in the subendothelial extracellular matrix: relation to tumor cell metastasis. Cancer Res *43*: 2704-2711, 1983.

17 Nakajima M, Irimura T, Di Ferrante D, Di Ferrante N and Nicolson GL: Heparan sulfate degradation: relation to tumor invasion and metastatic properties of mouse b16 melanoma sublines. Science *220*: 611-613, 1983.

18 Nakajima M, Irimura T, Di Ferrante N and Nicolson GL: Metastatic melanoma cell heparanase. Characterization of heparan sulfate degradation fragments produced by b16 melanoma endoglucuronidase. J Biol Chem *259*: 2283-2290, 1984.

19 Edovitsky E, Elkin M, Zcharia E, Peretz T and Vlodavsky I: Heparanase gene silencing, tumor invasiveness, angiogenesis, and metastasis. J Natl Cancer Inst *96*: 1219-1230, 2004.

20 Sato T, Yamaguchi A, Goi T, Hirono Y, Takeuchi K, Katayama K and Matsukawa S: Heparanase expression in human colorectal cancer and its relationship to tumor angiogenesis, hematogenous metastasis, and prognosis. J Surg Oncol *87*: 174-181, 2004.

21 Rohloff J, Zinke J, Choppmeyer K, Tannapfel A, Witzigmann H, Mössner J, Wittekind C and Caca K: Heparanase expression is a prognostic indicator for postoperative survival in pancreatic adenocarcinoma. Br J Cancer *86*: 1270-1275, 2002.

22 Takaoka M, Naomoto Y, Ohkawa T, Uetsuka H, Shirakawa Y, Uno F, Fujiwara T, Gunduz M, Nagatsuka H, Nakajima M, Tanaka N and Haisa M: Heparanase expression correlates with invasion and poor prognosis in gastric cancers. Lab Invest *83*: 613-622, 2003.

23 Finlay CA, Hinds PW, Tan TH, Eliyahu D, Oren M and Levine AJ: Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. Mol Cell Biol *8*: 531-539, 1988.

24 Wyllie AH, Bellamy CO, Bubb VJ, Clarke AR, Corbet S, Curtis L, Harrison DJ, Hooper ML, Toft N, Webb S and Bird CC: Apoptosis and carcinogenesis. Br J Cancer *80(Suppl 1)*: 34-37, 1999.

25 Grast-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W and Schulte-Hermann R: *In situ* detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology *21*: 1465-1468, 1995.

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