Modern Pathology (2011) 24, 1336–1347

Expression of HuR, COX-2, and survivin in lung cancers; cytoplasmic HuR stabilizes cyclooxygenase-2 in squamous cell carcinomas

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Hu antigen R (HuR) is a member of the human family of embryonic-lethal, abnormal vision-like proteins, which serves as an mRNA-binding protein. In the cytoplasm, HuR can stabilize the mRNA of cyclooxygenase-2 (COX-2), an enzyme that catalyses the synthesis of prostaglandins and is associated with promotion of carcinogenesis and tumor cell resistance to apoptosis. Intracellular (cytoplasmic and nuclear) localization of survivin has a prognostic significance as an apoptosis inhibitor and a regulator of cell division in tumors. Patients with 151 squamous cell carcinomas and 93 adenocarcinomas underwent lobectomy or pneumonectomy with hilar and mediastinal lymph node sampling. Paraffin-embedded tumor sections were retrieved for evaluation of nuclear and cytoplasmic staining of survivin and HuR, and cytoplasmic staining of COX-2. In squamous cell carcinomas, COX-2 expression was correlated with a difference of survivin (cytoplasmic−nuclear; $P=0.004$), cytoplasmic HuR ($P=0.018$), total HuR (cytoplasmic + nuclear; $P=0.009$), and difference of HuR ($P=0.020$). COX-2 was inversely correlated with nuclear survivin ($P=0.006$). In a univariate analysis by log-rank test, survival was associated with cytoplasmic survivin (adenocarcinoma, $P<0.001$; squamous cell carcinoma, $P=0.005$), difference of survivin (adenocarcinoma, $P<0.001$; squamous cell carcinoma, $P=0.014$), and COX-2 (squamous cell carcinoma, $P=0.001$). Survival was inversely associated with nuclear survivin (adenocarcinoma, $P=0.006$, squamous cell carcinoma, $P=0.014$). In a multivariate survival analysis, cytoplasmic survivin (adenocarcinoma, $P=0.002$; squamous cell carcinoma, $P=0.015$) and COX-2 (squamous cell carcinoma, $P=0.020$) were determined as independent prognostic factors. Cytoplasmic HuR expression is associated with COX-2 expression in squamous cell carcinomas. The expression of COX-2 in squamous cell carcinomas, and cytoplasmic survivin in adenocarcinomas and squamous cell carcinomas could be useful independent prognostic markers.
Modern Pathology (2011) **24,** 1336–1347; doi:10.1038/modpathol.2011.90; published online 13 May 2011

Keywords: apoptosis regulatory proteins; cyclooxygenase 2; immunohistochemistry; lung neoplasms; nuclear export signals; RNA-binding proteins

HuR (ELAVL1, the HUGO Gene Nomenclature Committee (HGNC)-approved official symbol for HuR) is an mRNA binding protein that belongs to the embryonic-lethal, abnormal vision-like protein family.[1] It binds to labile transcripts containing AU-rich elements, such as mRNAs for proto-oncogenes, cytokines, and cytokine-response genes. HuR is predominantly present in the nucleus where it can bind to target mRNAs; whereas in the cytoplasm, it stabilizes these messages.[2] HuR can stabilize cyclooxygenase-2 (COX-2) mRNA, leading to an increase in COX-2 expression (Figure 1).[3] There are some reports showing that the constitutive overexpression of COX-2 in ovarian and colon

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Received 12 October 2010; revised 21 March 2011; accepted 21 March 2011; published online 13 May 2011

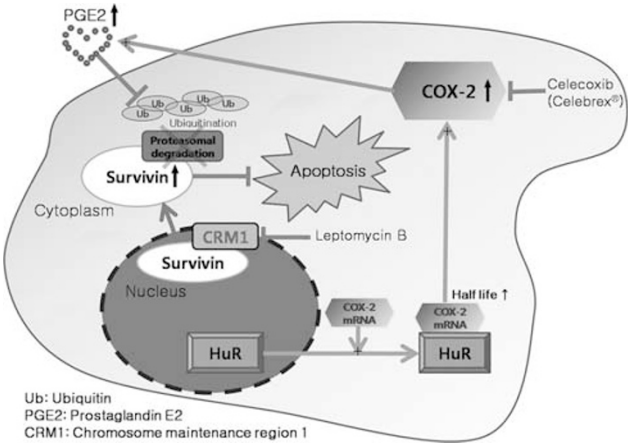

Figure 1 Diagram of HuR, COX-2, and survivin. HuR can bind to COX-2 mRNAs and stabilizes COX-2 mRNA, leading to an increase in COX-2 expression. COX-2 overexpression modulates survivin ubiquitination by a production of the COX-2 metabolite PGE_2. Survivin is detected in either the nucleus or the cytoplasm of cancer cells, or both subcellular pools. The nuclear export receptor Crm1 is involved in intracellular localization of survivin. Survivin is able to bind caspases, thus preventing apoptosis.

cancers is the result of HuR overexpression, and that this process has an important role in the carcinogenesis of several cancers.[3,4]

COXs are rate-limiting enzymes in prostanoid synthesis, which convert arachidonic acid into prostaglandin (PG) H2, a substrate for specific PG synthases.[5] Two isoforms of COX have been isolated and characterized: ubiquitously expressed COX-1 and inducible COX-2.[6] Studies of human cancers have revealed frequent overexpression of COX-2 in a variety of different malignancies, including lung cancers.[7–10] COX-2 (PTGS2, HGNC-approved official symbol for COX-2) and its enzymatic product PGE_2 have critical roles in inflammation and carcinogenesis, such as epithelial cell growth, invasion, and the promotion of tumor cell resistance to apoptosis (Figure 1).[11,12]

Survivin (BIRC5, HGNC-approved official symbol for survivin), a member of inhibitors of the apoptosis (IAP) gene family, is a 16.5-kD protein that inhibits apoptosis and regulates cell division. The expression of survivin is undetectable or found at very low levels in normal tissues, whereas it is found at high levels in embryonic and fetal tissues, and also various malignancies, including lung cancer.[13] Survivin is detected in either the nucleus or the cytoplasm of cancer cells, or both.[14] Recent evidence shows that the direct interaction of survivin with the nuclear export receptor Crm1 is critically involved in the intracellular localization and cancer-relevant functions of survivin.[15–18] Survivin is also able to bind caspases, thus preventing their activation (Figure 1).[19] Suppression of survivin expression leads to increased caspase-3 activation and apoptosis, as well as mitosis deregulation and sensitization to anticancer drugs.[20,21]

COX-2-dependent expression of survivin was previously reported as a critical factor in apoptosis resistance in non-small cell lung cancer cell lines.[22] However, the mechanism by which COX-2 exerts its cytoprotective effects is not completely understood. The relationship of nuclear versus cytoplasmic expression of survivin with apoptosis and the expression of HuR-related COX-2 expression is not well characterized in primary human lung cancers.[23–31]

In this study, we evaluated the expression of survivin, COX-2, and HuR, and subcellular localization of survivin and HuR in sets of pulmonary adenocarcinomas and squamous cell carcinomas patients, in relation to clinicopathologic parameters and survival. In addition, we investigated the relation of survivin, COX-2, and HuR expression.

Materials and methods

Patients and Specimens

A total of 244 patients with primary pulmonary adenocarcinomas and squamous cell carcinomas, diagnosed and operated on at Kyung Hee Medical Center, College of Medicine, Kyung Hee University, between 1985 and 2005, were included in this study. Within that sample were 151 squamous cell carcinomas and 93 adenocarcinomas. Patients underwent surgical resection with hilar and mediastinal lymph node sampling. Certain clinical and pathologic parameters were retrospectively summarized from the patient's file, including details on age, gender, histopathologic type, tumor differentiation, primary tumor, regional lymph node, distant metastasis and pathologic stage, and follow-up (Table 1). The mean of patient age at surgery was 62 years (range 35 to 81). Standard hematoxylin and eosin (H&E)-stained sections of the formalin-fixed, paraffin-embedded tumor tissues were reviewed by two pathologists (GY Kim and SJ Lim) to confirm the histologic diagnosis according to the current WHO classification.[32] Pathologic stage of disease in patients was determined according to the criteria set forth by the American Joint Committee on Cancer.[33]

Tissue Microarray

The paraffin-embedded tissues were sampled from archived conventional tissue blocks. Three areas of tumor were chosen by two surgical pathologists (GYK and SJL). The tissue microarrays were constructed with an AccuMax Array (ISU ABXIS/PeTagen) by sampling the three representative areas (2.0 mm in diameter) of the original tumor, and transferring them into a new array block.

Immunohistochemistry

Paraffin-embedded material was available in a set of 244 individual tumors for evaluation of nuclear and cytoplasmic staining of survivin and HuR, and

Table 1 Clinicopathologic characteristics of 93 ADCs and 151 SCCs of the lung

Characteristics	*ADC*		*SCC*	
	n = *93*	*%*	n = *151*	*%*
Gender				
Female	40	43	15	10
Male	53	57	136	90
Age (years)				
Range	35–81		37–81	
Mean (s.d.)	61.9 ± 9.4		61.9 ± 8.6	
Median	64		62	
Differentiation				
Well	9	10	8	5
Moderate	66	71	124	82
Poor	18	19	19	13
Primary tumor				
T1a	10	11	9	6
T1b	15	16	15	10
T2a	37	40	55	36
T2b	10	11	25	17
T3	16	17	35	23
T4	5	5	12	8
Regional LN				
N0	46	50	90	60
N1	17	18	37	24
N2	30	32	24	16
N3	0	0	0	0
Distant metastasis				
M0	90	97	151	100
M1a	3	3	0	0
M1b	0	0	0	0
Pathologic stage				
IA	18	19	17	11
IB	18	19	34	23
IIA	9	10	26	17
IIB	11	12	30	20
IIIA	31	33	42	28
IIIB	3	3	2	1
IV	3	3	0	0

ADC, adenocarcinoma; LN, lymph node; SCC, squamous cell carcinoma.

cytoplasmic staining of COX-2. Immunohistochemical staining was performed using 4-μm-thick tissue sections from the tissue microarray blocks and a Bond Polymer Intense Detection System (VisionBioSystems, Vic, Australia) according to the manufacturer's instructions, with minor modifications. In brief, each formalin-fixed and paraffin-embedded section was deparaffinized with Bond Dewax Solution (VisionBioSystems), and subjected to antigen retrieval using Bond ER Solution (VisionBioSystems) at 100°C for 30 min. The endogenous peroxidase was subsequently quenched by incubation with hydrogen peroxide for 5 min. The sections were then incubated for 15 min at room temperature, with anti-survivin (NB500-201; 1:1000; Novus, Littleton, CO, USA), anti-COX-2 (RM-9121-R7; 1:200; LabVision, Fremont, CA, USA), and anti-HuR antibodies (39-0600; 1:300; Zymed, Carlsbad, CA, USA), using a biotin-free polymeric horseradish peroxidase-linker antibody conjugate system in a Bond-maX automatic slide stainer (VisionBioSystems), and visualized using a 3.3-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCL buffer (pH 7.6), and 0.006% H_2O_2). The slides were counterstained with hematoxylin. Negative control slides without a primary antibody were included for each staining.

Immunohistochemical Evaluation

Staining was assessed in five high-powered fields at $\times 400$ magnification. Survivin and HuR immunoreactivity were evaluated semiquantitatively on the basis of staining intensity and proportion. The stained tumor tissues were scored blindly with respect to clinical patient by two investigators (GY Kim and SJ Lim). The cytoplasmic and nuclear staining patterns of survivin and HuR were evaluated separately. The proportion of staining was scored on a scale from 0 to 3 as follows: diffuse, >50% positive (score 3); regional, 25% to 50% positive (score 2); focal, 5 to 25% positive (score 1); negative (score 0). In addition, the intensity of staining was scored from 0 to 3 (0, absent; 1, weak; 2, moderate; 3, intense). The overall immunoreactive score for each sample was determined by multiplying the two individual scores. In order to describe the intracellular localization and expression levels of survivin and HuR in the tumors, total immunoreactive score (cytoplasmic immunoreactive score plus nuclear immunoreactive score) and the difference in immunoreactive score (cytoplasmic immunoreactive score minus nuclear immunoreactive score) were determined. Cytoplasmic and nuclear immunoreactive score were also further simplified in positive and negative cases as follows: moderate diffuse, intense regional, and intense diffuse were considered positive (high expression), and moderate regional, moderate focal, and all three proportions of weak staining were considered negative (low expression). Total immunoreactive score was divided into positive (≥ 12) and negative (<12). The difference in immunoreactive score was divided into positive (>0) and negative (≤ 0). The cut-off value was defined by ROC curve. COX-2 staining was scored as positive (>10%), and negative (0 to 10%).

Statistical Analysis

The remaining parameters were categorized as follows: age (<62 *vs* ≥ 62), histological grade (well to moderate *vs* poor), primary tumor (T1–T2 *vs* T3–T4), regional lymph node (N0 *vs* N1–N2), and pathologic stage (I–II *vs* III–IV). The relationships between the immunoreactive score of survivin,

COX-2, HuR, and the clinicopathologic parameters were investigated using Pearson's χ^2-test. Correlations of HuR *vs* COX-2, and COX-2 *vs* survivin were evaluated by the Spearman's rank correlation coefficient. Overall survival was calculated from the date of diagnosis to the date of death or last follow-up. Patients who were alive were censored at the time of their last follow-up visit. Deaths due to intercurrent causes were censored. Univariate analysis was performed, and the significance of differences in survival between the groups was determined using the log rank test. Cumulative survival curves and overall survival for groups were computed according to the Kaplan–Meier method. To evaluate the hazard ratios and the independent prognostic relevance, multivariate analysis using the Cox proportional hazard model was performed using the following covariates: sex, age, primary tumor, regional lymph node, distant metastasis, pathologic stage, and survivin, COX-2, and HuR levels, coded as they were in the univariate analysis. All tests of significance were two-sided, and differences were considered statistically significant at *P*-value of <0.05. Data analyses were performed using SPSS 15.0 (Chicago, IL, USA).

Results

Expressions of Survivin, COX-2, and HuR

As is shown in Table 2 and Figure 2, cytoplasmic, nuclear, difference, and the total survivin immunoreactivity were positive in 67 (72%), 62 (67%), 32 (34%), and 52 (56%) adenocarcinomas, respectively. In squamous cell carcinomas, cytoplasmic, nuclear, difference, and the total survivin immunoreactivity were positive in 116 (77%), 134 (89%), 18 (12%), and 112 (75%), respectively. COX-2 immunoreactivity was positive in 79 (85%) adenocarcinomas and 87 (58%) squamous cell carcinomas. Cytoplasmic, nuclear, difference, and the total HuR immunoreactivity were positive in 62 (67%), 87 (94%), 12 (13%), and 65 (70%) adenocarcinomas, and 103 (68%), 137 (91%), 24 (16%), and 115 (76%) squamous cell carcinomas, respectively.

Association of HuR, COX-2, and Survivin Expression with Clinicopathologic Variables

As is shown in Table 2, cytoplasmic survivin was found to be significantly increased in adenocarcinomas exhibiting poor differentiation ($P=0.024$) and higher stage of primary tumor ($P=0.030$), and in male patients with squamous cell carcinomas ($P=0.015$). Nuclear survivin expression of adenocarcinomas was found to be significantly increased in older patients ($P=0.015$), in patients with well differentiation ($P=0.042$) and a lower status of primary tumor ($P=0.015$). The difference in survivin expression of adenocarcinomas was found to be significantly increased in poor differentiation ($P=0.036$). Nuclear ($P<0.001$) and total ($P=0.003$) survivin expression were found to be significantly increased in squamous cell carcinomas. The difference of survivin ($P<0.001$) and COX-2 ($P<0.001$) expression were found to be significantly in creased in adenocarcinomas. Cytoplasmic HuR expression was found to be significantly increased in squamous cell carcinomas exhibiting poor differentiation ($P=0.044$). Nuclear ($P=0.035$) and total ($P=0.009$) HuR expression were found to be significantly increased in male patients with squamous cell carcinomas.

Correlations of HuR *vs* COX-2, and COX-2 *vs* Survivin Expression

In squamous cell carcinomas, cytoplasmic HuR ($\rho=0.192$, $P=0.018$), the difference in HuR ($\rho=0.190$, $P=0.020$), and total HuR ($\rho=0.212$, $P=0.009$), were positively correlated with COX-2 expression. COX-2 expression was positively correlated with the difference in survivin ($\rho=0.223$, $P=0.004$), and inversely correlated with nuclear survivin ($\rho=-0.221$, $P=0.006$; Table 3 and Figure 3).

Prognostic Value of Survivin, COX-2, and HuR Expression

The median survival of patients was determined as 53 ± 7.0 (adenocarcinoma) and 84 ± 23.3 months (squamous cell carcinoma) (Table 4). At the end of the study, 52 (56%) (adenocarcinoma) and 83 (55%) (squamous cell carcinoma) patients had died. The cumulative 5-year survival rate was 50% (adenocarcinoma) and 64% (squamous cell carcinoma). In a univariate analysis by log-rank test, primary tumor (T1–T2 *vs* T3–T4) (adenocarcinoma, $P<0.001$; squamous cell carcinoma, $P=0.001$), regional lymph node (N0 *vs* N1–N2) (adenocarcinoma, $P=0.040$; squamous cell carcinoma, $P=0.001$), distant metastasis (M0 *vs* M1) (adenocarcinoma, $P=0.028$), pathologic stage (I–II *vs* III–IV) (adenocarcinoma, $P=0.003$; squamous cell carcinoma, $P<0.001$), cytoplasmic survivin (adenocarcinoma, $P<0.001$; squamous cell carcinoma, $P=0.005$), the difference in survivin (adenocarcinoma, $P<0.001$; squamous cell carcinoma, $P=0.014$), and COX-2 levels (squamous cell carcinoma, $P=0.001$) were associated with shorter survival rate, whereas nuclear survivin was associated with a longer survival rate (adenocarcinoma, $P=0.001$, squamous cell carcinoma, $P=0.050$; Table 4). No significant correlation was found between HuR expression and survival. The overall survival for patients was statistically significant when described by Kaplan–Meier curves (Figure 4). In multivariate survival analysis using Cox proportional hazards model, cytoplasmic survivin (adenocarcinoma, $P=0.002$; squamous cell

Table 2 Correlations between expressions of survivin, Cox-2, and HuR with clinicopathologic characteristics in 93 ADCs and 151 SCCs of the lung

Characteristics	*Survivin (% within factor)*								*COX-2 (% within factor)*		*HuR (% within factor)*							
	C (high)		*N (high)*		*C−N>0*		*C+N≥12*		*Positive*		*C (high)*		*N (high)*		*C−N>0*		*C+N≥12*	
Histology	ADC	SCC	ADC	SCC	ADC	SCC	ADC	SCC	ADC	SCC	ADC	SCC	ADC	SCC	ADC	SCC	ADC	SCC
	67 (72)	116 (77)	62 (67)	134 (89)	32 (34)	18 (12)	52 (56)	112 (74)	79 (85)	87 (58)	62 (67)	103 (68)	87 (94)	137 (91)	12 (13)	24 (16)	65 (70)	115 (76)
P-value (χ^2)	0.403		**<0.001**		**<0.001**		**0.003**		**<0.001**		0.802		0.435		0.522		0.280	
Gender																		
Female	29 (73)	8 (53)	25 (63)	13 (87)	17 (43)	0 (0)	21 (53)	8 (53)	34 (85)	6 (40)	28 (70)	7 (47)	38 (95)	11 (73)	4 (10)	2 (13)	28 (70)	7 (47)
Male	37 (72)	108 (79)	37 (70)	121 (89)	15 (28)	18 (13)	31 (59)	104 (77)	45 (85)	81 (60)	34 (64)	96 (71)	49 (93)	126 (93)	8 (15)	22 (16)	37 (70)	108 (79)
P-value (χ^2)	0.932	**0.046**	0.459	0.678	0.154	0.218	0.565	0.065	0.990	0.146	0.554	0.079	0.696	**0.035**	0.468	1.000	0.984	**0.009**
Age																		
<62	25 (71)	56 (77)	18 (51)	63 (86)	16 (46)	10 (14)	16 (46)	53 (73)	29 (83)	37 (51)	21 (60)	51 (70)	31 (89)	67 (92)	5 (14)	10 (14)	22 (63)	57 (78)
≥62	42 (72)	60 (77)	44 (76)	71 (91)	16 (28)	8 (10)	36 (62)	59 (76)	50 (86)	50 (64)	41 (70)	52 (67)	56 (97)	70 (90)	7 (12)	14 (18)	43 (74)	58 (74)
P-value (χ^2)	0.918	0.976	**0.015**	0.359	0.075	0.514	0.124	0.670	0.662	0.095	0.289	0.673	0.193	0.666	0.759	0.475	0.251	0.592
Differentiation																		
Well	3 (33)	4 (50)	9 (100)	7 (88)	0 (0)	0 (0)	4 (44)	4 (50)	7 (78)	5 (63)	4 (44)	4 (50)	9 (100)	7 (88)	0 (0)	0 (0)	4 (44)	6 (75)
Moderate	49 (74)	96 (77)	40 (61)	109 (88)	27 (41)	17 (14)	36 (55)	93 (75)	58 (88)	71 (57)	44 (67)	90 (73)	60 (91)	114 (92)	9 (14)	22 (18)	46 (70)	98 (79)
Poor	15 (83)	16 (84)	13 (72)	18 (95)	5 (28)	1 (5)	12 (67)	15 (79)	14 (78)	11 (58)	14 (78)	9 (47)	18 (100)	16 (84)	3 (17)	2 (11)	15 (83)	11 (58)
P-value (χ^2)	**0.024**	0.170	**0.042**	0.766	**0.036**	0.507	0.525	0.331	0.321	1.000	0.210	**0.044**	0.491	0.407	0.541	0.542	0.132	0.126
Primary tumor																		
T1	14 (56)	19 (79)	22 (88)	21 (88)	4 (16)	3 (13)	15 (60)	20 (83)	23 (92)	18 (75)	17 (68)	19 (79)	23 (92)	23 (96)	4 (16)	6 (25)	17 (68)	21 (86)
T2	37 (78)	58 (73)	29 (62)	71 (89)	19 (40)	10 (13)	27 (57)	54 (68)	39 (83)	40 (50)	33 (70)	52 (65)	43 (92)	70 (88)	7 (15)	12 (15)	32 (68)	56 (70)
T3	14 (88)	29 (83)	7 (44)	31 (89)	8 (50)	4 (11)	7 (44)	28 (80)	13 (81)	21 (60)	10 (63)	24 (69)	16 (100)	32 (91)	1 (6)	6 (17)	12 (75)	29 (83)
T4	2 (40)	10 (83)	4 (80)	11 (92)	1 (20)	1 (8)	3 (60)	10 (83)	4 (80)	8 (67)	2 (40)	9 (67)	5 (100)	12 (100)	0 (0)	0 (0)	4 (80)	9 (75)
P-value (χ^2)	**0.030**	0.660	**0.015**	1.000	0.070	1.000	0.753	0.297	0.623	0.148	0.585	0.640	0.804	0.534	0.850	0.289	0.965	0.263
Regional LN																		
N0	31 (67)	70 (78)	34 (74)	80 (89)	14 (30)	13 (14)	25 (54)	66 (73)	39 (85)	47 (52)	31 (76)	64 (71)	42 (91)	80 (89)	7 (15)	15 (17)	32 (70)	68 (76)
N1	12 (82)	31 (84)	13 (77)	33 (89)	5 (29)	4 (11)	12 (71)	30 (81)	14 (82)	24 (65)	12 (71)	24 (65)	17 (100)	35 (95)	1 (6)	5 (14)	13 (77)	29 (78)
N2	22 (73)	15 (63)	15 (50)	21 (88)	13 (43)	1 (4)	15 (50)	16 (67)	16 (87)	16 (67)	19 (63)	15 (63)	28 (93)	22 (92)	4 (13)	4 (17)	20 (67)	18 (75)
P-value (χ^2)	0.531	0.148	0.062	1.000	0.456	0.459	0.376	0.436	0.923	0.263	0.870	0.637	0.650	0.679	0.658	0.905	0.779	0.934
Distant metastasis																		
M0	65 (72)	116 (77)	61 (60)	134 (89)	30 (33)	18 (12)	51 (57)	112 (74)	76 (84)	87 (58)	60 (67)	103 (68)	84 (93)	137 (91)	12 (13)	24 (16)	63 (70)	115 (76)
M1a	2 (67)	0 (0)	1 (33)	0 (0)	2 (67)	0 (0)	1 (33)	0 (0)	3 (100)	0 (0)	2 (67)	0 (0)	3 (100)	0 (0)	0 (0)	0 (0)	2 (67)	0 (0)
P-value (χ^2)	0.631	[a]	0.257	[a]	0.271	[a]	0.411	[a]	0.609	[a]	0.709	[a]	0.817	[a]	0.657	[a]	0.663	[a]
Pathologic stage																		
I	24 (67)	40 (78)	29 (81)	45 (88)	9 (25)	9 (18)	22 (61)	36 (71)	33 (92)	25 (49)	25 (69)	35 (69)	33 (92)	46 (90)	6 (17)	11 (22)	25 (69)	38 (75)
II	15 (75)	43 (77)	13 (65)	49 (87.5)	8 (40)	7 (13)	10 (50)	42 (75)	14 (70)	34 (61)	13 (65)	40 (71)	19 (95)	49 (88)	2 (10)	8 (14)	14 (70)	42 (75)
III	26 (77)	33 (75)	19 (56)	40 (91)	13 (38)	2 (5)	19 (56)	34 (77)	29 (85)	28 (64)	22 (65)	28 (64)	32 (94)	42 (96)	4 (12)	5 (11)	24 (71)	35 (80)
IV	2 (67)	0 (0)	1 (33)	0 (0)	2 (67)	0 (0)	1 (33)	0 (0)	3 (100)	0 (0)	2 (67)	0 (0)	3 (100)	0 (0)	0 (0)	0 (0)	2 (67)	0 (0)
P-value (χ^2)	0.787	0.925	0.073	0.899	0.300	0.143	0.721	0.747	0.195	0.299	0.946	0.706	1.000	0.385	0.859	0.366	1.000	0.820

ADC, adenocarcinoma; C, cytoplasmic; N, nuclear; C+N, total (sum) cytoplasmic and nuclear; C−N, difference between cytoplasmic and nuclear; LN, lymph node; SCC, squamous cell carcinoma.
[a]No statistics are computed because distant metastasis in SCC is a constant.
Bold values are statistically significant.

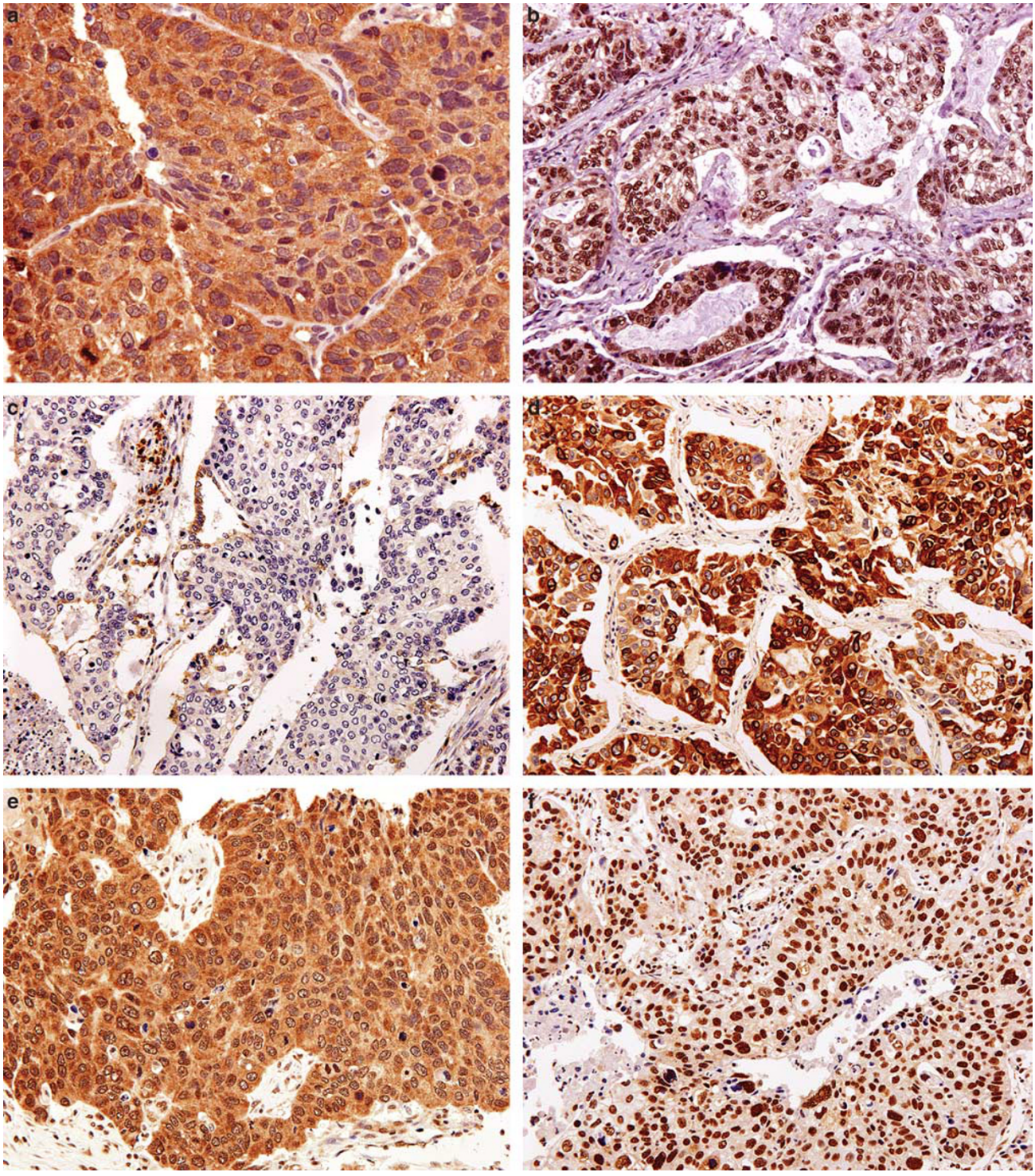

Figure 2 Immunohistochemical staining of survivin, COX-2, and HuR in non-small cell lung carcinomas. (**a**) Cytoplasmic survivin expression. (**b**) Nuclear survivin expression. (**c**) Negative expression of COX-2. (**d**) Positive expression of COX-2. (**e**) Cytoplasmic HuR expression. (**f**) Nuclear HuR expression. (Polymer method, **a**, ×400; **b** to **f**, ×200).

carcinoma, $P=0.015$) and COX-2 (squamous cell carcinoma, $P=0.020$) levels were determined as independent prognostic factors, along with primary tumor (T1–T2 *vs* T3–T4) (adenocarcinoma, $P<0.001$; squamous cell carcinoma, $P=0.002$; Table 5).

Discussion

HuR is predominantly present in the nucleus where it can bind to target mRNAs; in the cytoplasm, it stabilizes these messages.[2] HuR can stabilize COX-2 mRNA, leading to an increase in COX-2 expression.[3]

Table 3 Spearman's bivariate correlations of expression between COX-2 and survivin, or HuR in 93 ADCs and 151 SCCs of the lung

	COX-2			
	ADC		*SCC*	
	ρ *(rho)*	P-*value*	ρ *(rho)*	P-*value*
Survivin				
C	0.006	0.956	0.069	0.401
N	0.021	0.840	−0.221[a]	**0.006**
C−N	−0.012	0.912	0.223[a]	**0.004**
C+N	−0.010	0.921	−0.047	0.568
HuR				
C	0.085	0.418	0.192[b]	**0.018**
N	0.012	0.910	0.049	0.548
C−N	0.072	0.491	0.190[b]	**0.020**
C+N	0.117	0.264	0.212[a]	**0.009**

ADC, adenocarcinoma; C, cytoplasmic; N, nuclear; C+N, total (sum) cytoplasmic and nuclear; C−N, difference between cytoplasmic and nuclear; SCC, squamous cell carcinoma.
[a]Correlation is significant at the 0.01 level (two-tailed).
[b]Correlation is significant at the 0.05 level (two-tailed).
Bold values are statistically significant.

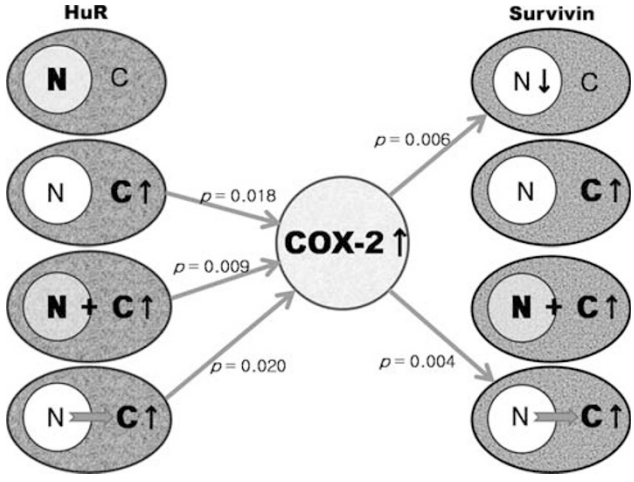

Figure 3 Correlations of HuR vs COX-2, and COX-2 vs survivin expression in 151 squamous cell carcinomas of the lung. Cytoplasmic HuR ($\rho = 0.192$, $P = 0.018$), total HuR ($\rho = 0.212$, $P = 0.009$), and difference of HuR ($\rho = 0.190$, $P = 0.020$) were positively correlated with COX-2 expression. COX-2 expression was positively correlated with survivin difference ($\rho = 0.223$, $P = 0.004$), and inversely correlated with nuclear survivin ($\rho = -0.221$, $P = 0.006$).

There have been some reports showing that the constitutive overexpression of COX-2 in ovarian and colon cancers is the result of HuR overexpression, and that this process has an important role in the carcinogenesis of several cancers.[3,4] A recent study reported that COX-2 expression is involved in COX-2 mRNA stabilization through HuR subcellular localization, and HuR abundance in a human lung epithelial cancer cell line, H460.[34] However, HuR expression related to COX-2 expression, and prognosis in primary human lung cancers has not been explored. In this study, we did not observe any significant correlations between HuR expression and pathologic stage, or survival. Our results did show that cytoplasmic HuR ($\rho = 0.192$, $P = 0.018$), the difference in HuR (cytoplasmic − nuclear; $\rho = 0.190$, $P = 0.020$), and total HuR (cytoplasmic + nuclear; $\rho = 0.212$, $P = 0.009$) were positively correlated with COX-2 expression in squamous cell carcinomas. These results suggest that increased expression of cytoplasmic HuR, independent of nuclear HuR levels, might contribute to the stabilization of COX-2 mRNA in squamous cell carcinomas of the lung. Furthermore, our study supports the suggestion that COX-2 could be a potential target of the mRNA-stabilizing activity of HuR, resulting in COX-2 overexpression.[4,35–39]

Elevated tumor COX-2 expression is associated with increased angiogenesis, tumor invasion, and promotion of tumor cell resistance to apoptosis. High level constitutive COX-2 expression has been detected in a variety of different malignancies, including lung cancers.[7–10] Non-steroidal anti-inflammatory drugs (NSAIDS), as well as specific COX-2 inhibitors, increase the susceptibility of cancer cells to apoptosis, and therefore, have been suggested both for cancer chemoprevention and therapy.[40] Previous studies have shown that COX-2 and its metabolite PGE_2 promote cancer cell survival.[41] COX-2 overexpression leads to survivin stabilization through decreased proteosomal degradation, thereby rendering the cancer cells more resistant to apoptotic stimuli.[12] The current results showed that increased COX-2 expression in squamous cell carcinomas was associated with shorter survival rate (log rank $P = 0.001$), and provide evidence for the importance of COX-2 overexpression in the regulation of anti-apoptotic proteins and lung cancer cell survival.

Survivin is overexpressed in several tumor types (including lung cancers) at both the protein and mRNA levels.[13] There is recent evidence that subcellular localization of survivin to the nuclear and cytoplasmic pools may also correlate with prognosis.[14] Nuclear and cytoplasmic pools of survivin have their distinct roles. Two functionally divergent splice variants of survivin have been indentified, survivin-2B and survivin-ΔEx3.[42] Survivin-ΔEx3 is found preferentially in the nucleus, whereas the wild type survivin and survivin-2B isoforms are localized in the cytoplasm. In the nucleus, survivin interacts with auroral B kinase and inner centromere protein (INCENP), and has a key role in completing mitosis. In the cytoplasm, survivin inhibits apoptosis by blocking caspase. It has been proposed that the nuclear pool of survivin is involved in promoting cell proliferation, whereas the cytoplasmic pool of survivin may participate in controlling cell survival, but not cell proliferation.[42,43] Recent evidence shows that the direct interaction of survivin with the nuclear export receptor Crm1 is critically involved in its intracellular localization and cancer-relevant

Table 4 Univariate analysis of the relationship between preoperative characteristics and survival in 93 ADCs and 151 SCCs of the lung

Characteristics	*No. of events (%)*		*Cumulative 5-year survival %*		*Median survival time (mo)* ± *s.e.*		*Log-rank* (P-*value)*	
	ADC	*SCC*	*ADC*	*SCC*	*ADC*	*SCC*	*ADC*	*SCC*
Overall	52 (56)	83 (55)	50.3	64.0	53 ± 7.0	84 ± 23.3	0.980	
Survivin								
C, low	5 (19)	16 (45)	79.3	73.6	210 ± 24.6	181 ± 41.2	**<0.001**	**0.005**
C, high	47 (70)	67 (57)	32.4	48.7	43 ± 3.9	48 ± 16.1		
N, low	25 (81)	11 (65)	22.9	28.9	37 ± 6.8	27 ± 6.4	**0.006**	**0.050**
N, high	27 (44)	72 (54)	57.1	58.3	106 ± 52.6	91 ± 24.6		
C−N ≤0	25 (41)	71 (53)	58.5	59.3	161 ± 60.8	97 ± 24.0	**<0.001**	**0.014**
C−N>0	27 (84)	12 (67)	21.4	21.7	32 ± 6.6	37 ± 9.2		
C+N<12	25 (41)	21 (54)	39.4	60.4	46 ± 4.4	173 ± 52.3	0.902	0.171
C+N≥12	27 (52)	62 (55)	48.0	53.3	58 ± 36.1	66 ± 20.6		
COX-2								
Negative	8 (57)	29 (45)	56.3	69.7	195 ± 185.0	181 ± 64.4	0.229	**0.001**
Positive	44 (56)	54 (62)	40.6	44.0	45 ± 5.9	39 ± 7.4		
HuR								
C, low	14 (45)	28 (58)	48.2	49.8	58 ± 24.1	48 ± 33.2	0.207	0.580
C, high	38 (61)	55 (53)	41.7	57.9	46 ± 5.0	91 ± 24.7		
N, low	4 (67)	8 (57)	66.7	50.0	66 ± 52.7	20 ± 39.8	0.789	0.616
N, high	48 (55)	75 (55)	41.5	55.6	46 ± 5.5	85 ± 25.0		
C−N ≤0	44 (54)	71 (56)	44.4	57.1	46 ± 8.6	80 ± 28.1	0.769	0.809
C−N>0	8 (67)	12 (50)	42.3	60.4	54 ± 9.7	85 ± 27.6		
C+N<12	14 (50)	19 (53)	54.8	54.5	66 ± 44.6	62 ± 43.5	0.192	0.942
C+N≥12	38 (58)	51 (56)	38.7	55.3	46 ± 4.8	85 ± 25.8		
Gender								
Female	23 (58)	9 (60)	41.7	42.9	45 ± 8.3	25 ± 19.6	0.761	0.774
Male	29 (55)	74 (54)	45.3	56.6	54 ± 12.2	92 ± 19.6		
Age								
<62	23 (66)	40 (55)	31.9	59.6	44 ± 2.6	106 ± 30.3	0.390	0.434
≥62	29 (50)	43 (55)	52.7	50.6	97 ± 39.9	66 ± 22.0		
Differentiation								
W-M	40 (53)	73 (55)	45.9	57.3	54 ± 11.9	97 ± 22.1	0.181	0.408
Poor	12 (67)	10 (56)	36.4	38.1	43 ± 9.6	38 ± 15.6		
Primary tumor								
T1–T2	36 (50)	50 (48)	52.3	63.0	72 ± 29.5	136 ± 49.9	**<0.001**	**0.001**
T3–T4	16 (76)	33 (70)	14.4	37.7	21 ± 8.1	27 ± 8.0		
Regional LN								
N0	22 (48)	41 (46)	62.5	62.6	97 ± 29.4	127 ± 28.3	**0.040**	**0.001**
N1–N2	30 (64)	42 (69)	27.3	44.7	41 ± 3.3	25 ± 18.6		
Distant metastasis								
M0	49 (54)	83 (55)	45.5	55.2	54 ± 10.0	84 ± 23.3	**0.028**	[a]
M1	3 (100)	0 (0)	0	[a]	29 ± 6.5	[a]		
Pathologic stage								
I–II	27 (48)	52 (49)	59.2	63.3	106 ± 46.5	127 ± 39.1	**0.003**	**<0.001**
III–IV	25 (68)	31 (71)	17.6	34.1	41 ± 6.5	24 ± 4.6		

ADC, adenocarcinoma; C, cytoplasmic; N, nuclear; C + N, total (sum) cytoplasmic and nuclear; C − N, difference between cytoplasmic and nuclear; LN, lymph node; SCC, squamous cell carcinoma; W-M, well to moderate.

[a]No statistics are computed because distant metastasis in SCC is a constant.

Bold values are statistically significant.

functions.[15–18] Nuclear export signals (NES) are leucine-rich, interact with the export receptor Crm1 in the nucleus, and depend on the RanGTP/GDP axis, which control the Crm1/substrate interaction.[16] Preferential nuclear survivin was indeed found to be a favorable predictor for various tumor types, although some reports consider nuclear survivin to be associated with poor survival.[43–45] In lung cancers, several studies indicated that expression of nuclear or cytoplasmic survivin is related to poor survival. Kren *et al*,[26] who evaluated only cytoplasmic survivin, identified it as a predictor of

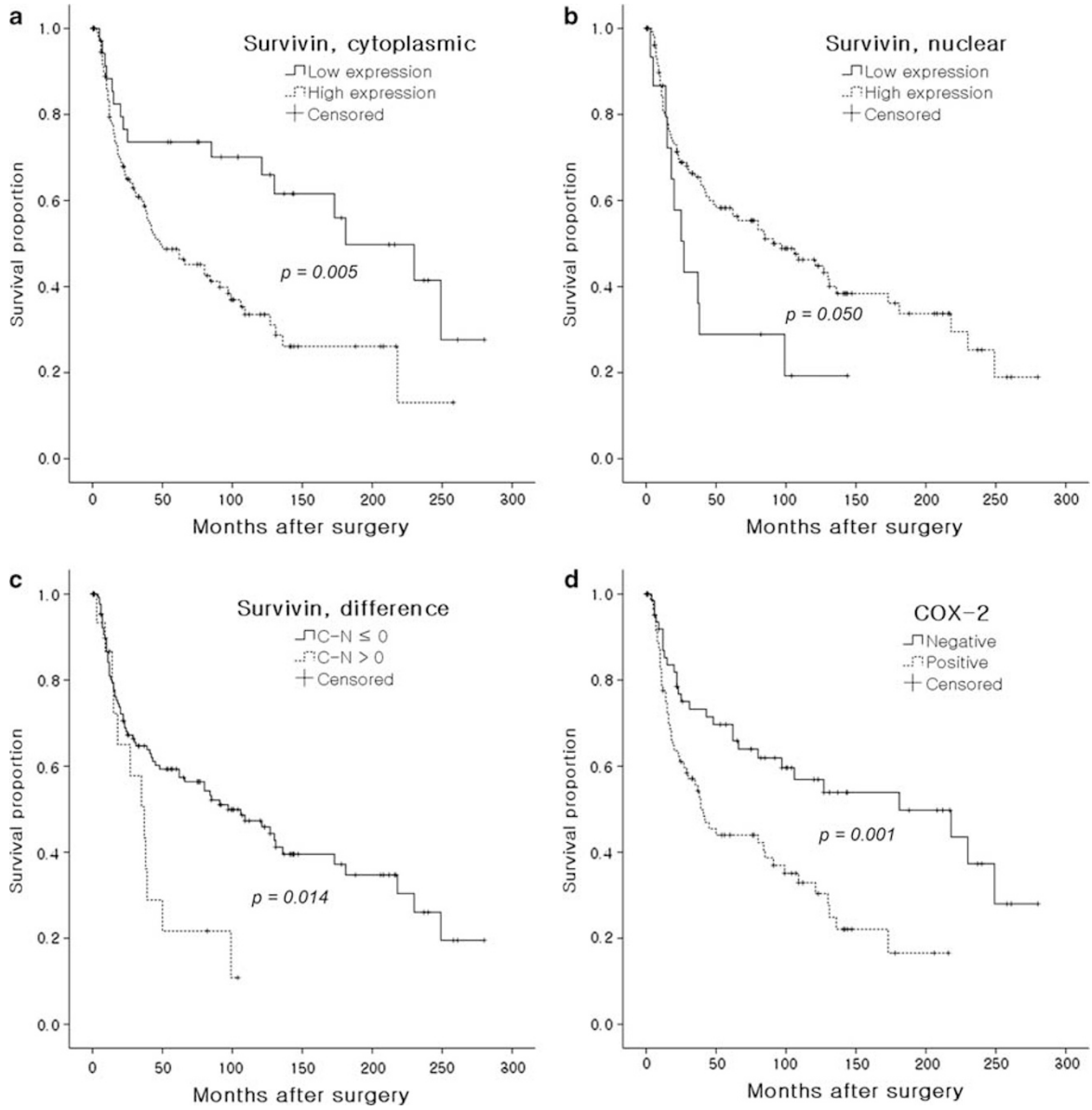

Figure 4 Kaplan–Meier survival curves showing overall survival of patients with squamous cell carcinomas of the lung. Patients are dichotomized according to the immunoreactive score. High expression of cytoplasmic survivin (**a**), low expression of nuclear survivin (**b**), difference (C−N > 0) between cytoplasmic and nuclear survivin (**c**), and positive COX-2 expression (**d**), are correlated with a shorter survival rate.

shorter survival, whereas Vischioni *et al*[24] identified nuclear survivin as a positive prognostic factor for survival. The results of this study, along with those of previous studies, on the location of survivin in cancer cells and its relation to survival and/or stage in pulmonary adenocarcinomas and squamous cell carcinomas are shown in Table 6. These seemingly contradictory findings regarding the relationship between survivin expression and prognosis may be explained in a few ways. First, the difference may be associated with the tumor types and/or the biopsies examined (pre-therapeutic *vs* post-therapeutic). Second, different commercial antibodies may have been used. Third, it may be due to the variable criteria used to classify a tumor as having more nuclear survivin or cytoplasmic survivin.[24,29,46] As the balance between cytoplasmic and nuclear survivin in tumor cells may be considered as an indicator for 'active survivin,' it is advisable to quantify not only absolute expression levels, but also the relative intracellular localization of survivin, cytoplasmic, nuclear, total (cytoplasmic + nuclear), and the difference in survivin expression (cytoplasmic − nuclear).[45] Our results showed that cytoplasmic survivin (log rank test, adenocarcinoma, $P<0.001$; squamous cell carcinoma, $P=0.005$) and the difference in survivin (log rank test, adenocarcinoma, $P<0.001$; squamous cell carcinoma, $P=0.014$) was associated with a poorer survival rate, whereas nuclear survivin expression (log rank

Table 5 COX proportional hazards model in 93 ADCs and 151 SCCs of the lung

Variables	*Level*	*Histology*	*OR*	*95% CI*	P-*value*
Gender	Female *vs* male	ADC	0.944	0.500–1.784	0.860
		SCC	0.618	0.298–1.282	0.197
Age (years)	< 62 *vs* ≥ 62	ADC	0.723	0.394–1.327	0.295
		SCC	1.186	0.750–1.877	0.466
Differentiation	W-M *vs* P	ADC	1.622	0.823–3.195	0.162
		SCC	1.125	0.550–2.301	0.747
Primary tumor	T1–T2 *vs* T3–T4	ADC	3.018	1.628–5.598	**<0.001**
		SCC	2.016	1.285–3.162	**0.002**
Regional LN	N0 *vs* N1–N2	ADC	1.717	0.976–3.022	0.061
		SCC	2.052	1.316–3.200	**0.002**
Distant metastasis	M0 *vs* M1	ADC	1.231	0.332–4.566	0.756
		SCC	[a]	[a]	[a]
Pathologic stage	I–II *vs* III–IV	ADC	1.183	0.360–3.890	0.782
		SCC	1.459	0.758–2.809	0.258
Survivin					
C	Low *vs* high	ADC	4.514	1.708–11.929	**0.002**
		SCC	2.052	1.148–3.575	**0.015**
N	Low *vs* high	ADC	0.505	0.088–2.892	0.443
		SCC	0.962	0.313–2.961	0.947
C−N	≤ 0 *vs* >0	ADC	1.794	0.994–3.239	0.053
		SCC	1.702	0.878–3.299	0.115
C+N	< 12 *vs* ≥ 12	ADC	2.192	0.856–5.610	0.102
		SCC	1.026	0.340–3.098	0.964
COX-2	Low *vs* high	ADC	1.755	0.747–4.124	0.197
		SCC	1.777	1.095–2.883	**0.020**
HuR					
C	Low *vs* high	ADC	1.254	0.396–3.971	0.700
		SCC	0.780	0.463–1.314	0.351
N	Low *vs* high	ADC	1.307	0.239–7.148	0.757
		SCC	0.576	0.274–1.211	0.146
C−N	≤ 0 *vs* >0	ADC	0.678	0.296–1.553	0.358
		SCC	0.657	0.344–1.254	0.203
C+N	<12 *vs* ≥ 12	ADC	0.840	0.415–1.698	0.627
		SCC	1.001	0.440–2.281	0.998

ADC, adenocarcinoma; C, cytoplasmic; N, nuclear; C + N, total (sum) cytoplasmic and nuclear; C − N, difference between cytoplasmic and nuclear; CI, confidence interval; P, poor; OR, odds ratio; SCC, squamous cell carcinoma; W-M, well to moderate.

[a]No statistics are computed because distant metastasis in SCC is a constant.

Bold values are statistically significant.

Table 6 Nuclear and/or cytoplasmic survivin immunohistochemistry, and its relation with stage and prognosis of pulmonary ADCs and SCCs in other studies and the present study

Author	*Antibody*	*No. of cases*	*Stage*	*Survival*	*Year* [reference]
Falleni	Polyclonal NB 500-201 (ab469); Novus	44	C, +	Not related	2003 [23]
Vischioni	Polyclonal NB 500-201 (ab469); Novus	53	—	N, good	2004 [24]
Lu	Monclonal D8 (SC-17779); Santa Cruz	48	—	N, poor	2004 [25]
Kren	Polyclonal goat anti-human; Santa Cruz	102	C, +	C, poor	2004 [26]
Shinohara	Monclonal D8 (SC-17779); Santa Cruz	41	—	N, poor	2005 [27]
Atikcan	Monoclonal 4F7; Neo Markers	58	Not related	N, poor	2006 [28]
Ulukus	Monoclonal 4F7; Neo Markers	63	N, −	Not related	2007 [29]
Bria	Polyclonal (ab469); Abcam	116	—	N, poor	2008 [30]
He	Polyclonal (ZA-0458); Zhongshan Goldenbridge	51	C, + C and N, +	N, good	2009 [31]
Present study	Polyclonal NB 500-201 (ab469); Novus	244	Not related	N, good C, poor	—

C, cytoplasmic immnunoreactivity; N, nuclear immunoreactivity; '+', correlated; '−', = inversely correlated; '—', not evaluated.

test, adenocarcinoma, $P = 0.006$; squamous cell carcinoma, $P = 0.050$) was associated with a favorable survival rate. Even though the above factors were not associated with a high cancer stage, these results are consistent with proposed hypothesis and findings in previous studies.[24,26,45] The current data suggest that cytoplasmic and active survivin (survivin converted from nuclear to cytoplasmic) represents 'cytoprotective survivin' in tumor cells, whereas nuclear survivin may have 'impaired survivin function.'

Our results show that COX-2 expression in squamous cell carcinomas was positively correlated

with the difference in survivin level (cytoplasmic immunoreactive score—nuclear immunoreactive score; $\rho = 0.223$, $P = 0.004$), and inversely correlated with nuclear survivin ($\rho = -0.221$, $P = 0.006$). These findings suggest that active ('dynamic') survivin exported from the nucleus to the cytoplasm through an NES/Crm1 transporter is significantly associated with increased expression of COX-2. Thus, increased expression of active cytoplasmic survivin and decreased nuclear survivin could be an indicator of the inhibition of apoptosis of tumor cells or tumor progression. Our results support the hypothesis that COX-2 overexpression and PGE_2 overproduction inhibit survivin ubiquitination, which leads to its stabilization and prevents proteasomal degradation of survivin in squamous cell carcinoma cells. Our findings provide evidence for the importance of COX-2 overexpression in the regulation of anti-apoptotic proteins and lung cancer cell survival. In addition, these results suggest that NES/Crm1 transporter should be pursued as a potential target of tumor therapy. To our knowledge, the present study is the first large scale study for evaluation of HuR, COX-2, and survivin expression and its correlations in primary adenocarcinomas and squamous cell carcinomas of human lung.

In conclusion, cytoplasmic HuR expression is associated with COX-2 expression in pulmonary squamous cell carcinomas. Our study supports the suggestion that COX-2 could be a potential target of the mRNA-stabilizing activity of cytoplasmic HuR, resulting in COX-2 overexpression. Increased COX-2 expression is associated with active conversion of survivin from nuclear to cytoplasmic, and low expression of nuclear survivin, which contributes to the anti-apoptotic effect and over proliferation of pulmonary squamous cell carcinomas. The expression of COX-2 and cytoplasmic survivin in pulmonary squamous cell carcinomas is associated with poor survival rate and could be a useful, independent prognostic marker, and potential therapeutic strategy for pulmonary squamous cell carcinomas.

Acknowledgement

This work was supported by a grant from the Kyung Hee University Research Fund in 2006 (KHU-20061223).

Disclosure/conflict of interest

The authors declare no conflict of interest.

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