Livin and caspase-3 expression are negatively correlated in cervical squamous cell cancer

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Summary

Objective: Overexpression in cancer cells of inhibitor of apoptosis proteins like livin appears to promote tumorigenesis by regulating expression of proteins involved in apoptosis signaling. Here, the authors investigated expression of livin and an apoptosis protein that is known to inhibit, caspase-3, in cervical squamous cell carcinoma. *Materials and Methods:* Their expression was assessed for correlation with tumor invasiveness. Immunohistochemistry for livin and caspase-3 was used in 36 normal cervical tissues and in 98 samples of cervical squamous cell carcinoma. The percentage of cells expressing these proteins was compared between normal and cancer samples. Their expression rates in cancer samples were subsequently compared with one another and with the clinical and pathological characteristics of the samples. *Results:* Livin was more commonly expressed in tumor samples than in normal tissues, while the opposite pattern was observed for caspase-3. Expression of livin was significantly associated with advanced clinical stage, higher pathological grade, and lymph node metastasis ($p < 0.05$). Expression of caspase-3 was significantly associated with lower clinical stage, lower pathological grade, and lack of lymph node metastasis ($p < 0.05$). Finally, expression of livin was negatively correlated to caspase-3 expression in cervical squamous cell carcinoma tissue ($r = -0.57$, $p < 0.05$). *Conclusions:* Livin may inhibit apoptosis in cervical squamous cell carcinoma by downregulating caspase-3, thereby promoting disease progression.

Key words: Livin; Caspase-3; Cervical squamous cell carcinoma; Immunohistochemtry.

Introduction

Although the incidence has declined in recent years, cervical cancer remains a common malignant tumor in women. Cervical cancer occurs in two main pathological types, squamous cell carcinoma and adenocarcinoma; 80%-90% of cervical cancers present as squamous cell carcinoma [1]. The disease is typically caused by the human papillomavirus (HPV), which can activate oncogenes such as p16 [2] and p53 [3], thereby causing cell proliferation and cell apotosis disorders [4] that promote tumorigenesis.

Research efforts have been devoted to understanding how proteins involved in apoptosis become dysregulated in cancer cells. In particular, inhibitors of apoptosis (IAPs) appear to play important roles in preventing apoptosis by binding to caspases, the activators of programmed cell death, allowing tumor cells to avoid elimination. Expression of several IAPs is higher in tumor cells than in normal tissues. One IAP, livin (also known as ML-IAP), is expressed in normal fetal kidney and liver and adult testis and thymus, but is also highly-expressed in lymphoma [5], melanoma cell lines, breast [6], and esophageal carcinoma [7]. Livin inhibits apoptosis by binding caspases 3, 7, and 9 [8]. Caspase-3 is a significant executioner in the caspase family because it hydrolyzes a number of substrates [9]. This protein initiates cell death following activation by either the mitochondrial or the death ligand pathway, after which the caspase cascade is triggered [10].

Given the interaction between livin and caspase-3 and the role of IAPs like livin in tumorigenesis, the authors sought to determine whether expression of livin is dysregulated in cervical squamous cell carcinoma, whether its expression effected a change in caspase-3, and whether these changes may correspond to disease severity. To determine the relationship between livin and caspase-3, immunohistochemistry was applied to these two proteins in tissue samples from normal and cancerous cervixes. Protein expression was also correlated to tumor pathology.

Materials and Methods

Specimens

Cervical tissues were collected from 134 patients who received biopsy or surgery treatment in Department of Obstetrics and Gynecology in the first People's Hospital of Yancheng City from June 2009 to June 2011 and had not received any radiotherapy and chemotherapy. These samples included 98 cases of cervical squamous cell carcinoma and 36 normal cervical tissues. Of the cancer patients, 45 women were less than 45 years old, and 53 were greater than or equal to 45 years old. Samples were clinically classified according to the International Federation of Gynecology and Obstetrics (FIGO): 61 cases were Stage I, and 37 cases were Stage II. Pathological classification [11] indicated 14 well-differentiated cases (low-grade), 46 moderately-differentiated cases, and 38 poorly-differentiated (high-grade) cases. Additionally, 31 cases had lymph node

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Table 1. — *Expression of livin and caspase-3 proteins in normal and squamous cell carcinoma cervical tissues.*

	Livin						Caspase-3				
	n	–	+	++	+++	Percent cells positive (%)	–	+	++	+++	Percent cells positive (%)
Normal cervical tissue	36	32	2	1	1	11.1	10	6	9	11	72.2
Cervical squamous cell cancer	98	21	35	24	18	78.6	72	7	9	10	26.5
χ^2	50.1						23.2				
p	< 0.05						< 0.05				

Table 2. — *Livin and caspase-3 expression correlate with clinico-pathological parameters.*

	n	Livin				p	Caspase-3				p
		–	+	++	+++		–	+	++	+++	
Age (years)											
< 45	45	9	16	10	10	> 0.05	32	3	4	6	> 0.05
≥ 45	53	12	19	14	8		40	4	5	4	
FIGO clinical classification											
Stage I	61	17	18	14	12	< 0.05	39	5	8	9	< 0.05
Stage II	37	4	17	10	6		33	2	1	1	
Pathological grade											
Well-differentiated	14	12	1	1	0	< 0.05	4	3	5	2	< 0.05
Moderately-differentiated	45	5	21	14	5		35	2	3	5	
Poorly-differentiated	39	4	13	9	13		33	2	1	3	
Lymph node metastasis											
Yes	31	2	12	9	8	< 0.05	28	1	2	0	< 0.05
No	67	19	23	15	10		44	6	7	10	

metastasis and 67 cases did not. All pathology specimens were fixed with four percent paraformaldehyde, paraffin-embedded, and sectioned at five μm thickness.

Immunohistochemistry

Paraffin sections were dewaxed and re-hydrated. Antigens were repaired in tissue by microwave heat for five min, and endogenous peroxidase was blocked with incubation for 10 min in three percent hydrogen peroxide. Sections were soaked for 20 min in five percent normal rabbit serum, then treated with rabbit anti-human livin antibody (1:100) and rabbit anti-human caspase-3 antibody (1:200). Sections were incubated with primary antibodies overnight at room temperature, then washed with 0.05% phosphate-buffered saline Tween-20 (PBST, pH 7.4). Goat anti-rabbit IgG/HRP (1:100) secondary antibody was incubated with sections for 40 min at room temperature. Sections were washed again with PBST. Vectashield Elite-AP alkaline phosphatase substrate was applied to sections for 30 min, then sections were washed with PBST before detection with DAB chromogen for 20 min. Tissue was counterstained with hematoxylin prior to visualizaiton under light microscopy.

Staining assessment

Staining for livin and caspase-3 indicated localization of both proteins to the endochylema. Five visual fields were randomly selected at high magnification. The proportion of positively-stained cells in the total number of cells was multiplied by 100 to determine percent positive. Fewer than 5% positive for livin or caspase-3 staining was scored as (-), between 6% and 25% positive was scored as (+), between 26% and 50% was scored as (++), and > 50% positive was scored as (+++). "Positive" is used in the results to describe scores of +, ++, and +++.

Statistical analysis

SPSS13.0 statistical software was used for statistical analysis. The chi-squared (χ^2) test and Wilcoxon rank sum test were used to compare each set of samples. Spearman rank correlation test was used to analyze the relationship between livin and caspase-3. A p value < 0.05 was considered statistically significant.

Results

Expression of livin and caspase-3 in cervical carcinoma

Expression of livin was compared between 98 squamous cell carcinoma and 36 normal cervical tissues. In normal cervix, only 4/36 samples (11.1%) exhibited livin expression in more than 5% of cells. However, 77/98 (78.6%) cervical carcinoma samples were positive for livin expression (Table 1). This difference in livin expression between normal and squamous cell carcinoma cervical tissues was significantly different ($\chi^2 = 50.1$, $p < 0.05$). In contrast, caspase-3 was more likely to be detected in normal cervix than in cervical cancer: 26/36 (72.2%) of normal tissue samples were positive for caspase-3 expression and just 26/98 (26.5%) cervical squamous cell cancer tissues were positive for caspase-3 (Table 1). Thus, the differences in expression of caspase-3 between normal and cancerous cervical tissues were statistically significant ($\chi^2 = 23.2$, $p < 0.05$).

Livin and caspase-3 expression are differentially correlated with clinico-pathological parameters of cervical squamous cell carcinoma

Although expression of livin in cervical squamous cell cancer was not correlated with patient age, its expression was more prevalent in Stage II vs Stage I tumors ($p < 0.05$; Table 2). Similarly, livin expression was lower in well-differentiated tumors than in moderately- and poorly-differentiated tumors, and thus its expression was correlated with higher tumor grade ($p < 0.05$). Livin expression was also higher in tumors with lymph node metastasis than those without lymph node metastasis ($p < 0.05$).

Age was also not a factor for caspase-3 expression in cervical squamous cell cancer. In contrast to livin expres-

sion, caspase-3 expression was more common in Stage I cervical squamous cell cancer than in Stage II ($p < 0.05$; Table 2). Additionally, correlations of caspase-3 with pathological grade were identified: expression of caspase-3 was more common in well-differentiated than in moderately- or poorly-differentiated tumors and, therefore, caspase-3 expression was correlated with lower tumor grade ($p < 0.05$). Finally, caspase-3 expression in cervical squamous cell cancer was correlated with not having lymph node metastasis as it was more commonly detected in tumors that had not metastasized ($p < 0.05$).

Livin and caspase-3 expression correlation in cervical squamous cell cancer

In cervical squamous cell cancer tissues, livin was expressed in 78.6% of tumors, while caspase-3 was expressed in 26.5%. Spearman rank correlation test indicated that the expression of these proteins in cervical squamous cell tumors was negatively-correlated (Table 3).

Table 3. — *Correlation of livin and caspase-3 expression in cervical squamous cell cancer.*

Livin	Caspase-3		Total
	Positive	Positive	
Negative	11	66	77
Negative	15	6	21
Total	26	72	98
r		– 0.57	
p		< 0.05	

Discussion

The occurrence of cervical squamous cell cancer is not only related to HPV infection, but also to the imbalance of carcinogen-induced cell proliferation and apoptosis that follows HPV infection. Cell apoptosis initiates in response to intrinsic or extrinsic factors via a signaling program [12]. Several important players in this program are the caspase family proteins [13], Bcl-2 family proteins [14], p53 [15], and survivin [16].

The inhibition of apoptosis can allow cancer cells to escape elimination, promoting tumorigenesis. IAP proteins have therefore become central to the investigation of cancer mechanisms. In particular, the IAP protein livin is important for its ability to inhibit apoptosis by interacting with caspases, especially caspase-3. Recent work has demonstrated that livin expression is dysregulated in tumors, such that it is more abundantly expressed in the tumor cells [6,7]. Additionally, Gazzaniga *et al*. showed that, in bladder cancer, the postoperative recurrence time of patients with expression of livin in their tumors was shorter compared to patients lacking livin expression; thus, livin expression is correlated with poorer prognosis [17]. The present research results were consistent with those obtained by Gazzaniga *et al*. Livin was significantly more commonly expressed in cervical squamous cell carcinoma than in normal cervical tissues. Further, expression of livin in tumors was significantly correlated with higher cancer Stage (clinical classification, $p < 0.05$), worse pathological grade (differentiation degree, $p < 0.05$), and lymph node metastasis ($p < 0.05$). Thus, expression of livin protein can predict poorer prognosis in patients with cervical squamous cell carcinoma.

Of the caspase family proteases involved in apoptosis, caspase-3 is one of the most significant. In cases in which caspase-3 expression is dysregulated (i.e., expression is reduced), apoptosis may not occur at a normal level. Indeed, in tumor cells, caspase-3 expression is down-regulated. The authors investigated whether caspase-3 expression is dysregulated in cervical squamous cell carcinoma. They found that caspase-3 appears to be down-regulated in these tumors; expression was more common in normal cervical tissues than in the carcinoma tissues. Additionally, caspase-3 expression was correlated with lower cancer Stage (clinical classification; $p < 0.05$), lower pathological grade (differentiation degree, $p < 0.05$), and lack of lymph node metastasis ($p < 0.05$). Thus, reduced or absent caspase-3 expression can predict a poorer prognosis in patients with cervical squamous cell carcinoma. These results are consistent with reports from ovarian cancer [18] and breast cancer [19].

Finally, the authors determined whether livin and caspase-3 expression were correlated with one another. Indeed, these proteins displayed a negative correlation of expression in cervical squamous cell cancer, suggesting that livin may regulate and control cell apoptosis by inhibiting expression of caspase-3. In fact, recent work indicates that livin reduces apoptosis by directly decreasing expression of caspase-3 and inhibiting death acceptor pathways. Furthermore, livin can inhibit mitochondrial apoptosis pathways by combining with caspase-9 [20].

In summary, the authors found that that livin is highly-expressed in cervical squamous cell cancer tissues, and its expression level is related to disease severity. In contrast, caspase-3 is minimally expressed in cervical squamous cell cancer tissues, and its expression level is inversely related to disease severity. The negative correlation between livin and caspase-3 expression suggests that livin expression can promote cervical squamous cell cancer by inhibiting cellular apoptosis.

References

[1] Vesco K.K., Whitlock E.P., Eder M., Burda B.U., Senger C.A., Lutz K.: "Risk factors and other epidemiologic considerations for cervical cancer screening: a narrative review for the U.S. Preventive Services Task Force". *Ann. Intern. Med*., 2011, *155*, 698.

[2] Fonseca-Silva T., Farias L.C., Cardoso C.M., Souza L.R., Carvalho Fraga C.A., Oliveira M.V. *et al*.: "Analysis of p16 (CDKN2A) methylation and HPV-16 infection in oral mucosal dysplasia". *Pathobiology*, 2012, *79*, 94.

[3] Ernoux-Neufcoeur P., Arafa M., Decaestecker C., Duray A., Remmelink M., Leroy X. *et al*.: "Combined analysis of HPV DNA, p16, p21 and p53 to predict prognosis in patients with Stage IV hypopharyngeal carcinoma". *J. Cancer Res. Clin. Oncol*., 2011, *137*, 173.

[4] Anderson L.A.: "Prophylactic human papillomavirus vaccines: past, present and future". *Pathology*, 2012, *44*, 1.

[5] Yang D., Song X., Zhang J., Ye L., Wang S., Che X., *et al*.: "Therapeutic potential of siRNA-mediated combined knockdown of the IAP genes (Livin, XIAP, and Survivin) on human bladder cancer T24 cells". *Acta Biochim. Biophys. Sin*. (Shanghai) 2010, *42*, 137.

[6] Liang C.Y., Ma P., Liu X.L.: "RNAi-mediated gene silencing of livin synergistic with epirubicin enhance apoptosis of human breast cancer cells". *Zhonghua Yi Xue Za Zhi*, 2008, *88*, 1703.
[7] Chen L., Ren G.S., Li F., Sun S.Q.: "Expression of livin and vascular endothelial growth factor in different clinical stages of human esophageal carcinoma". *World J. Gastroenterol*., 2008, *14*, 5749.
[8] Abd-Elrahman I., Hershko K., Neuman T., Nachmias B., Perlman R., Ben-Yehuda D.: "The inhibitor of apoptosis protein Livin (ML-IAP) plays a dual role in tumorigenicity". *Cancer Res*., 2009, *69*, 5475.
[9] Chougule M.B., Patel A.R., Jackson T., Singh M.: "Antitumor activity of Noscapine in combination with Doxorubicin in triple negative breast cancer". *PloS One*, 2011, *6*, 17733.
[10] Tong J., Xie G., He J., Li J., Pan F., Liang H.: "Synergistic antitumor effect of dichloroacetate in combination with 5-fluorouracil in colorectal cancer". *J. Biomed. Biotechnol*., 2011, *2011*, 740564. Epub 2011, Feb. 20.
[11] Gok M., Rozendaal L., Berkhof J., Visser O., Meijer C.J., van Kemenade F.J.: "Cytology history preceding cervical cancer diagnosis: a regional analysis of 286 cases". *Br. J. Cancer*, 2011, *104*, 685.
[12] O'Neal P., Alamdari N., Smith I., Poylin V., Menconi M., Hasselgren P.O.: "Experimental hyperthyroidism in rats increases the expression of the ubiquitin ligases atrogin-1 and MuRF1 and stimulates multiple proteolytic pathways in skeletal muscle". *J. Cell. Biochem*., 2009, *108*, 963.
[13] Han Y., Gao L., Sun N., Zhang J., Zhang W., Zhou X., *et al*.: "Effects of Kang-Jia-Wan, a Chinese medicinal herb officinal, on apoptosis induction in goiter of rats". *J. Ethnopharmacol*., 2009, *122*, 533.
[14] Onori P., DeMorrow S., Gaudio E., Franchitto A., Mancinelli R., Venter J., *et al*.: "Caffeic acid phenethyl ester decreases cholangiocarcinoma growth by inhibition of NF-kappaB and induction of apoptosis". *Int. J. Cancer*, 2009, *125*, 565.
[15] Qiu W., Wu B., Wang X., Buchanan M.E., Regueiro M.D., Hartman D.J. *et al*.: "PUMA-mediated intestinal epithelial apoptosis contributes to ulcerative colitis in humans and mice". *J. Clin. Invest*., 2011, *121*, 1722.
[16] Chan K.S., Wong C.H., Huang Y.F., Li H.Y.: "Survivin withdrawal by nuclear export failure as a physiological switch to commit cells to apoptosis". *Cell. Death. Dis*., 2010, *1*, 57.
[17] Gazzaniga P., Gradilone A., Giuliani L., Gandini O., Silvestri I., Nofroni I., *et al*.: "Expression and prognostic significance of LIVIN, SURVIVIN and other apoptosis-related genes in the progression of superficial bladder cancer". *Ann. Oncol*., 2003, *14*, 85.
[18] Kumar S., Bryant C.S., Chamala S., Qazi A., Seward S., Pal J., *et al*.: "Ritonavir blocks AKT signaling, activates apoptosis and inhibits migration and invasion in ovarian cancer cells". *Mol. Cancer*, 2009, *8*, 26.
[19] Scarlatti F., Maffei R., Beau I., Codogno P., Ghidoni R.: "Role of non-canonical Beclin 1-independent autophagy in cell death induced by resveratrol in human breast cancer cells". *Cell. Death. Differ*., 2008, *15*, 1318.
[20] Yang D., Song X., Zhang J., Ye L., Wang S., Che X, *et al*.: "Suppression of livin gene expression by siRNA leads to growth inhibition and apoptosis induction in human bladder cancer T24 cells". *Biosci. Biotechnol. Biochem*., 2010, *74*, 1039.

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