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Survivin and Fas Ligand Expressions Are Correlated with Angiolymphatic Tumor Spread in Medullary Thyroid Carcinoma

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Background : Medullary thyroid carcinoma (MTC) that originates from C cells comprises about 10% of all the malignant thyroid tumors. Activating mutations of the RET proto-oncogene have been found to be involved in the anti-apoptotic pathway of MTC that harbors the RET mutation. We investigated the correlation between the clinicopathologic parameters and the expressions of survivin, a novel anti-apoptotic molecule, and the other apoptosis-related proteins, and the known prognostic markers. **Methods :** Immunohistochemical staining was performed using antibodies for survivin, Fas, Fas ligand (FasL), bcl-2, calcitonin, CEA and cyclin A in 19 case of MTC; 10 sporadic MTCs, eight multiple endocrine neoplasia (MEN) type 2A MTCs and one familial MTC (FMTC). **Results :** Survivin protein expression was found in five cases (26%) and this was correlated with the presence of angiolymphatic tumor emboli (p=0.019). FasL was expressed in 14 cases (74%) and it had correlation with the presence of lymph node metastases (p=0.029). The cyclin A-labeling indices were correlated with local invasiveness (p=0.001). **Conclusions :** Survivin and FasL might be involved in the lymphatic tumor spread of MTC.

Key Words : Survivin protein; Thyroid gland; Carcinoma, medullary

Medullary thyroid carcinoma (MTC) is a malignant tumor that originates from calcitonin cells (C-cells) of the thyroid and it comprises about 10% of all the malignant thyroid tumors. Up to 25% of MTCs are heritable and they are caused by germline gain of function mutations in the RET (REarranged during Transfection) proto-oncogene that has autosomal-dominant inheritance and high penetrance.[1,2] Multiple endocrine neoplasia (MEN) 2A, MEN 2B and familial MTC (FMTC) are included in this heritable group. Oncogenic RET mutations mediate the up-regulation of NF-kB activity, and this is known to be involved in the anti-apoptotic pathway of the MTC harboring RET mutation.[3]

Neoplastic C-cell hyperplasia is regarded as a precursor lesion of heritable MTC and it usually occurs at a very young age. So it is difficult to explain why rapid tumor progression is rarely identified before the third or fourth decades of life.[4,5] It has not been clarified whether the germline mutation itself in the RET proto-oncogene is sufficient or not for the development and progression of MTC.

Somatic RET mutations are also found in 8-80% of sporadic MTCs, depending on the study and the method used to detect the mutation,[6,7] but the genotypes of sporadic MTCs are not well associated with their metastatic outcome.[8] The etiology of sporadic MTC is still unknown.

The recently cloned survivin gene is a member of the inhibitors of apoptosis (IAP) family; it located at 17q25 and it comprises three introns and four exons encoding 142 amino acids in human, and this survivin protein inhibits apoptosis through a pathway that is different from that involving the bcl-2 family.[9] Survivin specifically inhibits the processing of the downstream effectors of apoptosis, i.e., caspase-3 and caspase-7, in the cells receiving

apoptotic stimulation from such things as tumor necrosis factor (TNF)-α, chemotherapeutic drugs, viral infections and oxidative stress.[10,11]

There have been only a few studies about the apoptotic or anti-apoptotic molecules in MTC. The down-regulation of bcl-2 was noted in the more aggressive MTC group.[12,13] Fas ligand (FasL) was detected in 30% of MTC at a low intensity.[14] However, there has been no report about the survivin expression in MTC.

In the present study, we investigated the expression rate of survivin and the other apoptosis-related proteins including Fas, FasL and bcl-2 in MTCs. We analyzed the clinicopathologic correlation of the expression levels of these apoptosis-related proteins as well as other candidate prognostic factors such as calcitonin, CEA and cyclin A.

MATERIALS AND METHODS

Patients and tissues

Nineteen patients with MTC underwent thyroidectomy from 1994 to 2000, and their tumor specimens were retrieved from the archives of the department of pathology at the Seoul National University Hospital. The original hematoxylin & eosin-stained slides were reviewed, and we reconfirmed the original diagnoses. The most representative and well preserved, formalin-fixed, paraffin-embedded tumor blocks from each case were used for the immunohistochemical staining.

Clinicopathologic evaluation

Ten patients with sporadic MTC had no familial MTC history, no other neoplasm of the MEN syndrome or no neoplastic C-cell hyperplasia in their resected thyroid. Eight patients with MEN 2A syndrome had a history of operation for unilateral or bilateral adrenal pheochromocytomas and parathyroid hyperplasia or adenoma. One patients with FMTC had a history of MTCs in his mother, older brother and aunt.

We evaluated the various clinicopathologic parameters including age, gender, tumor size, the status of local invasion, lymph node metastasis, TNM stage and angiolymphatic tumor emboli by reviewing the patients' medical records and the original H&E slides. The TNM stage was classified according to the 2004 World Health Organization classification of endocrine tumors.[2]

Immunohistochemistry

The representative formalin-fixed, paraffin-embedded blocks were cut into 4 μm-thick sections. The sections were then dried, deparaffinized and rehydrated according to the usual procedure. For antigen retrieval, the slides were incubated in 0.1 M of citric acid buffer at pH6.0 for 15 min by using microwave oven. The slides were treated with 0.3% H_2O_2 in methanol for 30 min to abolish the endogenous peroxidase activity. To detect the survivin, the sections were blocked with 1% goat serum in PBS and then incubated overnight with anti-survivin polyclonal antibody (RD system, Minneapolis, USA, dilution 1:1,500) at 4℃. The sections were incubated with biotinylated anti-rabbit IgG antibody, and this was followed by incubation with avidin-biotin-peroxidase complex. They were developed in a substrate solution of 0.01% diaminobenzidine-hydrogen peroxide and counterstained with Harris hematoxylin. For the detection of Fas, FasL, bcl-2, calcitonin, CEA and cyclin A, immunohistochemical stainings were carried out in a Ventana ES automated immunohistochemistry system (Ventana Medical Systems, Tucson, AZ) and by using a basic diaminobenzidine detection kit (Ventana Medical Systems, Tucson, AZ). Polyclonal antibodies for Fas (C20, Santa Cruz, California, USA, dilution 1:20) and FasL (N20, Santa Cruz, California, USA, dilution 1:100), and monoclonal antibodies for bcl-2 (Zymed, California, USA, dilution 1:50), calcitonin (Novocastra, Nottinghill, UK, dilution 1:100), CEA (Dako, Glostrup, Denmark, dilution, 1:100), and cyclin A (Novocastra, Nottinghill, UK, dilution, 1:20) were used as the primary antibodies. Normal saline was used instead of the primary antibody for a negative control.

Two pathologists assessed the immunohistochemical staining semiquantitatively. For survivin, Fas, FasL, bcl-2, calcitonin and CEA, no staining or rare (<5%) immunopositive cells were considered as a negative result. Stainings in 5% to 30%, more than 30% upto 50%, and in more than 50% of tumor cells were interpreted as mild, moderate and strong positive expressions, respectively. For cyclin A, the labeling index was obtained by manual counting of more than 1,000 tumor cells in high power fields.

Statistical analysis

The data were analyzed by T-test, Mann-Whitney U test, Jonckheere-Terpstra test and Kruskal Wallis test using SPSS 11.0 software. p values less than 0.05 were considered as statistically significant.

Table 1. Clinicopathologic parameters of medullary thyroid carcinomas

Case No.	Age (years)	Sex	Group	pT	pN	pM	Stage	Size (mm)	Perithyroid invasion	AngLym emboli	F/up
1	49	Female	Sporadic	2	0	0	II	30	–	–	NED
2	38	Female	Sporadic	3	0	0	III	25	+	–	NED
3	36	Male	Sporadic	1	1a	0	III	20	–	+	NED
4	32	Female	Sporadic	1	0	0	I	15	–	–	NED
5	70	Male	Sporadic	3	0	0	III	40	+	–	LR, M[a]
6	64	Female	Sporadic	3	0	0	III	50	+	+	NED
7	29	Female	Sporadic	2	1b	0	IVA	30	–	–	NED
8	71	Male	Sporadic	3	1b	0	IVA	50	+	+	NED
9	52	Female	Sporadic	1	1a	0	III	20	–	–	NED
10	41	Female	Sporadic	1	0	0	I	17	–	–	NED
11	43	Female	MEN2A	3	0	0	III	25	+	–	NED
12	19	Female	MEN2A	3	1b	0	IVA	50	–	+	LNM
13	52	Male	MEN2A	1	1b	0	IVA	17	–	–	LNM
14	29	Female	MEN2A	2	0	0	II	25	–	–	LR, LNM
15	41	Female	MEN2A	1	1b	0	IVA	7	–	–	NED
16	17	Male	MEN2A	2	0	0	II	25	–	–	NED
17	40	Male	MEN2A	1	0	0	I	20	–	+	NED
18	14	Female	MEN2A	1	0	0	I	7	–	–	NED
19	25	Male	FMTC	1	0	0	I	10	–	–	NED

AngLym, angiolymphatic; F/up, follow-up; NED, no evidence of disease; LR, local recurrence; M[a], metastasis to spine; LNM, regional LN metastasis.

Table 2. Immunohistochemical analysis of medullary thyroid carcinomas

Case	Group	Survivin	Fas	FasL	bcl-2	Calcitonin	CEA	Cyclin A LI
1	Sporadic	–	++	+	+	+++	+++	1.00
2	Sporadic	–	+++	+	+++	–	–	1.70
3	Sporadic	+	++	++	+	+++	+++	0.75
4	Sporadic	–	–	–	–	++	+	0.00
5	Sporadic	–	+++	+	+++	+	–	1.57
6	Sporadic	–	–	–	++	+++	+++	3.40
7	Sporadic	–	+++	++	+++	+++	+++	0.70
8	Sporadic	+	++	+	+	++	+++	1.75
9	Sporadic	–	++	+	++	+++	++	0.33
10	Sporadic	–	+	+	+++	+++	+++	1.17
11	MEN2A	–	–	–	+++	+++	+++	0.40
12	MEN2A	++	+	++	+++	+++	+++	0.48
13	MEN2A	–	+++	+	++	+++	+++	0.40
14	MEN2A	++	+	+	++	+++	+++	0.33
15	MEN2A	–	+++	+	++	+++	+++	0.49
16	MEN2A	–	++	–	–	–	–	0.72
17	MEN2A	+++	++	++	+++	+++	+++	0.81
18	MEN2A	–	+	–	+++	+++	+++	0.63
19	FMTC	–	++	+	+++	+++	+++	0.40

LI, labeling index; +, mild; ++, moderate; +++, strong positive reaction.

RESULTS

Analysis of the clinocopathologic parameters

The clinicopathologic data of the selected cases are summarized in Table 1. The mean age of the MEN 2A/FMTC group was 31.1 years (range: 14 to 52 years) and the mean age of the sporadic group was 48.2 years (range: 29 to 71 years; p=0.042). The other clinicopathologic parameters such as gender, tumor size, perithyroidal invasion, angiolymphatic tumor emboli, TNM stage and the rate of tumor recurrence or metastasis during the follow-up period were not significantly different between the sporadic and MEN 2A/FMTC groups.

Immunohistochemical analysis

The results and the representative features of the immunohistochemical stainings are shown in Table 2 and Fig. 1, respectively. There were no significant differences in the expression rates of survivin, Fas, FasL, bcl-2, calcitonin, CEA and cyclin A between the sporadic MTC and the MEN 2A/FMTC groups.

Survivin protein expression was found in five cases (26%) and this showed statistically significant correlation with the presence of angiolymphatic tumor emboli (p=0.019; Table 3). FasL expression was found in 14 cases (74%) and it had a meaningful correlation with the presence of lymph node metastases (p=0.029). FasL also had positive correlation with the expression of survivin (p=0.014). Fas and bcl-2 were expressed in 16 cases (84%) and 17 cases (89%), respectively, but both Fas and bcl-2 did not show any correlation with the clinicopathologic parameters or expressions of other apoptosis-related proteins. Calcitonin and CEA were strongly expressed in 17 (89%) and 16 cases (84%), respec-

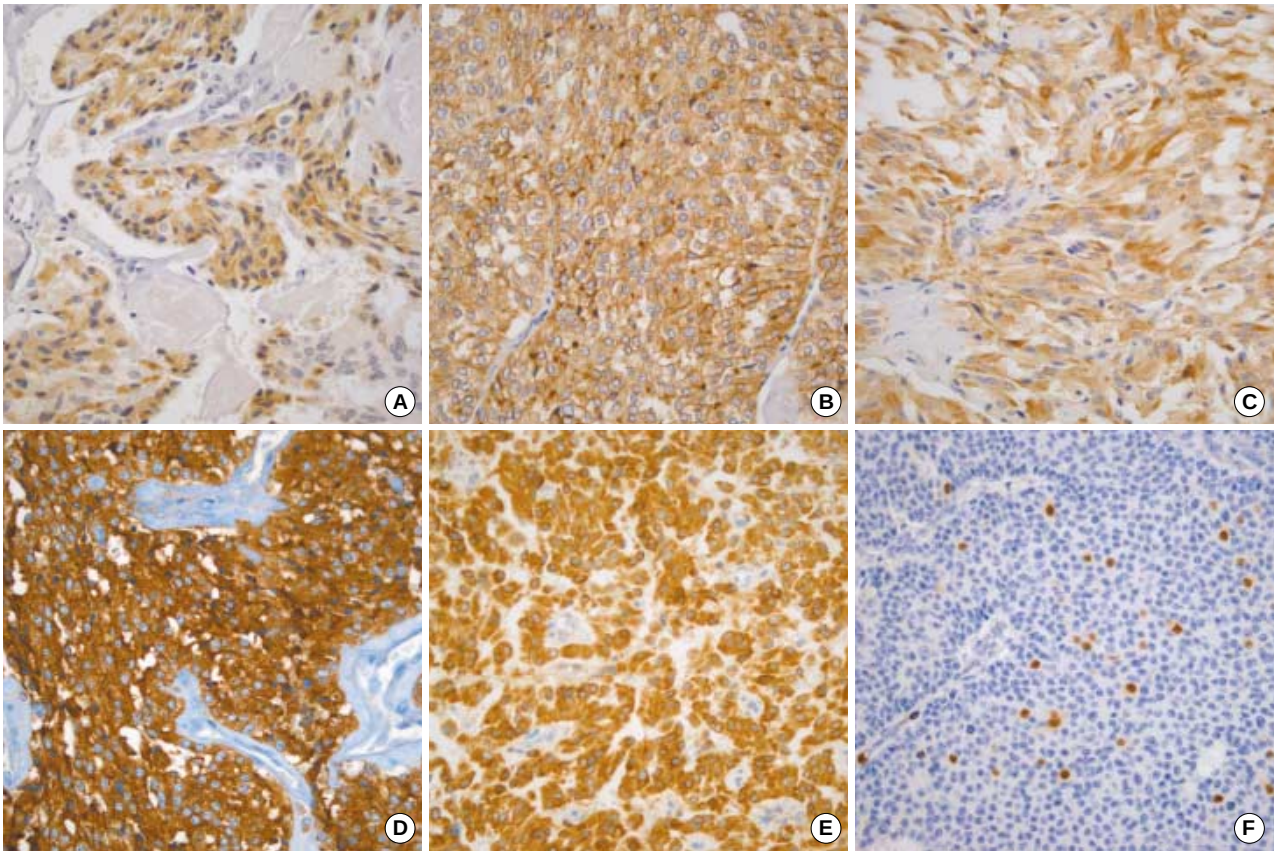

Fig. 1. Immunohistochemical stains of medullary thyroid carcinomas. (A) Survivin is expressed in cytoplasm of tumor cells. (B) Fas expression of tumor cells is mainly membranous and cytoplasmic patterns. (C) FasL is also expressed in cytoplasmic and membranous patterns. (D) CEA is strongly expressed in the cytoplasm in the majority of tumor cells. (E) Bcl-2 is expressed in membranous and cytoplasmic patterns in the majority of tumor cells. (F) Cyclin A-positive individual tumor cells are shown.

Table 3. Correlation between clinicopatholgic parameters and immuohistochemical results

Variables		Total cases	Survivin		p value	FasL		p value	Cyclin A	p value
			negative n (%)	positive n (%)		negative n (%)	positive n (%)		LI (Mean±SD)	
Perithyroid invasion	Absent	14	10 (71.4)	4 (28.6)	NS	11 (78.6)	3 (21.4)	NS	0.58±0.29	0.001
	Present	5	4 (80.0)	1 (20.0)		(60.0)	2 (40.0)		1.76±0.95	
LN metastasis	Absent	12	10 (83.3)	2 (16.7)	NS	7 (58.3)	5 (41.7)	0.029	1.01±0.87	NS
	Present	7	4 (57.1)	3 (42.9)		7 (100)	0 (0.0)		0.70±0.45	
AngLym Emboli	Absent	14	13 (92.9)	1 (7.1)	0.019	10 (71.4)	4 (28.6)	NS	0.70±0.47	NS
	Present	5	1 (20.0)	4 (80.0)		4 (80.0)	1 (20.0)		1.44±1.07	

LN, lymph node; AngLym, angiolymphatic; LI, labeling index; SD, standard deviation, NS, not significant.

tively, yet the calcitonin and CEA expressions did not correlate with any of the clinicopathologic parameters or the other apoptosis-related proteins expressions. MTC usually showed rare mitotic activity. Although the cyclin A-labeling indices were only 0 to 3.4%, they were correlated with the presence of local invasion into the perithyroidal soft tissue (p=0.001).

DISCUSSION

In general, tumorigenesis is regulated by the balance between the cell proliferation and apoptosis. Apoptosis is also thought to be involved in the metastatic mechanism as well as in the transformation into more aggressive tumor. Invasion and metastasis of tumor cells require the cell's ability to escape apoptosis under the condition of loss of normal contact with the extracel-

lular matrix (ECM).[15] The ECM normally affects cell behavior and it plays an important role in the regulation of cellular morphogenesis, differentiation, transformation and growth via triggering the signal transduction pathway.[16] So, the tumor cell clones that have a more aggressiveness or metastatic potential should be armed with anti-apoptotic protective mechanisms to escape their anchorage dependency with the ECM.

The apoptotic process may be principally initiated by signals from two distinct pathways: the intrinsic or mitochondrial pathway and the extrinsic or receptor-mediated pathway. The intrinsic pathway is regulated by members of the bcl family, in which the bcl-2 proto-oncogene is the prototype that inhibits the apoptosis induced by various stimuli such as growth factor or survival signal deprivation.[17] The extrinsic pathway is initiated by engagement of the TNF receptor family such as the Fas and TNF receptor. FasL cross-linked with Fas activates the cytoplasmic death domain in the Fas bearing cells and this results in the cleavage cascade of the cystein proteases.[18] The Fas-FasL system is well known to be involved in the apoptosis of activated T cells, B cells and natural killer cells. Induction or up-regulation of functional FasL has been suggested as a candidate mechanism for immune evasion in various human neoplasms including malignant melanoma, lung cancer and colorectal cancer.[19]

Hinze *et al*.[20] reported that the bcl-2 level was clearly diminished in non-neoplastic C-cell hyperplasia in comparison with high bcl-2 expression seen in most neoplastic C-cell hyperplasia as well as in hereditary MTCs, except for some undifferentiated ones. Their results suggest that bcl-2 alteration is a very early event in the development of MTC and it may have a role in the dedifferentiation process. In the present study, bcl-2 was positive at more than a moderate degree in 14 cases (73.7%), but it was weakly positive in three cases (15.8%), and completely negative in only two cases (10.5%). There was no prognostic significance for the bcl-2 protein expression level, which was different from the results of a previous study that showed bcl-2 heralded a poor prognosis.[12] Further large-scale studies with long-term follow-up need to be conducted on bcl-2 protein expression as an independent prognostic marker.

Whereas recombinant tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induced apoptosis in most thyroid carcinoma cell lines of a follicular cell origin, the MTC cell line was resistant to Fas-induced apoptosis and TRAIL-induced apoptosis, and this cell line was even resistant to transfection of constitutively active caspase-8.[21] That report suggested that a different regulation of the apoptotic pathway might be involved in MTC carcinogenesis.

Compared with the two relatively well investigated pathways of apoptosis that were mentioned above, the molecular machinery of the anti-apoptotic mechanism of the IAP family has not been elucidated. The IAP family has been found in virus, fly, yeast, protozoa and mammals, and in common it has a baculoviral IAP-repeat (BIR) domain, a caspase recruitment domain (CARD), a ubiquitin-conjugating (UBC) domain and a carboxy-terminal RING zinc-finger domain.[10] Survivin is the smallest member of the IAP family that contains only one copy of the BIR domain, which is thought to be essential for apoptosis inhibition.[9,22,23] It has been suggested that survivin counteracts a default induction of apoptosis in the G2-M phase of the cell cycle via interaction with the mitotic spindle microtubules.[24] In the previous studies, survivin was found to be constitutively expressed in human fetal tissues including kidney, liver, lung and brain, but not in the terminally differentiated adult tissues except for thymus, testis and placenta tissue.[9,25] The over-expression of survivin in human cancers has been reported to correlate with poor survival for patients with neuroblastoma, stomach cancers, breast cancers and colorectal cancers.[26-29]

In the present study, the expression of survivin protein was noted in 26% of the MTCs and it was correlated with the presence of angiolymphatic tumor emboli. Moreover the expression of FasL, a candidate molecule of immune evasion, was correlated with the presence of lymph node metastasis. Although it is unclear whether organ-specificity exists or not, these results imply that anti-apoptotic molecules such as survivin and FasL might play important roles in MTC for the acquisition of metastatic potential via the lymphatics. These anti-apoptotic molecules may provide a potentially therapeutic target for treating MTC in the near future.

During the follow-up period that ranged from 2 to 8 years, the rates for local recurrence and for remote or regional lymph node metastases were not significantly correlated with the expression of survivin or FasL. Because the 5 and 10-year survival rates of MTC are relatively favorable at about 83.2% and 73.7%, respectively,[30] more long-term follow-up data will be required to determine the biologic significance of the expressions of these anti-apoptotic molecules, survivin and FasL.

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