

# Peptidergic innervation of human esophageal and cardiac carcinoma

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## Abstract

**AIM:** To investigate the distribution of neuropeptide-immunoreactive nerve fibers in esophageal and cardiac carcinoma as well as their relationship with tumor cells so as to explore if there is nerve innervation in esophageal and cardiac carcinoma.

**METHODS:** Esophageal and cardiac carcinoma specimens were collected from surgical operation. One part of them were fixed immediately with 4 % paraformaldehyde and then cut with a cryostat into 40- $\mu$ m-thick sections to perform immunohistochemical analysis. Antibodies of ten kinds of neuropeptide including calcitonin gene-related peptide (CGRP), galanin (GAL), substance P (SP), etc. were used for immunostaining of nerve fibers. The other part of the tumor specimens were cut into little blocks (1 mm<sup>3</sup>) and co-cultured with chick embryo dorsal root ganglia (DRG) to investigate if the tumor blocks could induce the neurons of DRG to extend processes, so as to probe into the possible reasons for the nerve fibers growing into tumors.

**RESULTS:** Substantial amounts of neuropeptide including GAL-, NPY-, SP-immunoreactive nerve bundles and scattered nerve fibers were distributed in esophageal and cardiac carcinomas. The scattered nerve fibers waved their way among tumor cells and contacted with tumor cells closely. Some of them even encircled tumor cells. There were many varicosities aligned on the nerve fibers like beads. They were also closely related to tumor cells. In the co-culture group, about 63 % and 67 % of DRG co-cultured with esophageal and cardiac tumor blocks respectively extended enormous processes, especially on the side adjacent to the tumor, whereas in the control group (without tumor blocks), no processes grew out.

**CONCLUSION:** Esophageal and cardiac carcinomas may be innervated by peptidergic nerve fibers, and they can induce neurons of DRG to extend processes *in vitro*.

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## INTRODUCTION

The problem about the innervation of tumors was noticed as

early as 50 years ago. Though many authors at that time studied this problem and some obtained affirmative results, the ultimate conclusion made by authoritative pathologist<sup>[1]</sup> was that there was no innervation in tumor. And this theory was been generally accepted since then.

However, because of the involvement of mental factors in the genesis and development of tumors, the studies on the relationship between nerve and tumor never stopped. Some authors<sup>[2,3]</sup> claimed their findings of nerve fibers in tumors, and some others<sup>[4]</sup> studied the perineural invasion by carcinoma cells. In our previous investigations, we also observed many scattered nerve fibers in meningioma and cardiac cancer, which was closely related to tumor cells. In addition, accumulated data<sup>[5-7]</sup> show that neuropeptides are involved in the regulation of the growth of tumors. Considering these new advancements, we decided to study the distribution of neuropeptide-containing nerve fibers in esophageal and cardiac carcinomas as well as their relationship with tumors so as to find some new proofs about tumor innervation.

## MATERIALS AND METHODS

### Reagents

Antibodies of ten kinds of neuropeptide were obtained from Sigma, Chemicon or Bohringmanham Companies. Their working dilutions are listed in Table 1. ABC kit was obtained from Vector Company.

**Table 1** Antibodies of neuropeptides and their work dilution

Antibody	Working dilution
CGRP	1:8 000
GAL	1:8 000
SP	1:5 000
NT	1:4 000
SOM	1:8 000
CCK	1:4 000
L-ENK	1:5 000
Dyn	1:4 000
NPY	1:6 000
M-ENK	1:6 000

CGRP: Calcitonin gene-related peptide, GAL: Galanin, SP: Substance P, NT: Neurotensin, SOM: Somatostatin, CCK: Cholecystokinin, L-ENK: Leu-enkephalin, Dyn: Dynorphine, NPY: Neuropeptide Y, M-ENK: Met-enkephalin.

### Specimens

The surgical tumor specimens were collected from the Affiliated Cancer Hospital of Beijing University and the General Hospital of People's Liberation Army. Clinical and pathological diagnoses confirmed that the esophageal carcinoma specimens were squamous cell carcinomas and cardiac carcinoma specimens were adenocarcinomas. In our preliminary investigations, we found that the probability to find neuropeptide immunoreactive nerve fibers was decreased in large tumors, only tumors with diameter under 3 cm were selected for the present study. Totally, 16 cases of esophageal carcinoma and 13 cardiac carcinoma specimens were used.

After rinsed in saline, one part of the tissue were fixed immediately by immersing in 4 % paraformaldehyde in 0.01 M PBS (pH 7.4) for 10 hrs at 4 °C and then in PBS containing 30 % sucrose to stay overnight. The specimens were then cut with a cryostat (JUNG 2700, America) into 40- $\mu$ m-thick sections for immunohistochemical analysis. The other part of the specimens were collected into DMEM containing 1 000U/ml penicillin and streptomycin for co-culture experiment.

### Immunohistochemistry

Immunohistochemical reactions were carried out according to the avidin-biotinylated peroxidase complex method (ABC) as previously reported<sup>[8]</sup>. Briefly, the tumor sections were incubated at room temperature for 30 min in 1.2 % H<sub>2</sub>O<sub>2</sub> (v/v) solution to quench endogenous peroxidase activity. After washed with PBS for 10 min $\times$ 3, they were incubated with 0.3 % Triton X-100 and then moved into antibodies of neuropeptides (diluted by PBS containing 1 % BSA) for 24-48 hrs at 4 °C. The sections were then incubated in biotin-conjugated IgG (diluted in PBS, 1:200) for 2.5hrs at room temperature and washed for 30 min by three changes of PBS, followed by incubation with the streptavidin-peroxidase complex for 2.5 hrs. The reaction product was detected with 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma). Nerve fibers containing the neuropeptides were identified by the presence of dark black color. Thereafter, part of the sections were counterstained with neutral red and dehydrated in graded ethanol before mounted.

Negative controls were made either by omitting the specific neuropeptide antibodies or by replacing them with non-immune goat serum to assess the nonspecific adsorption of secondary antibodies.

### Co-culture of tumor blocks with DRG

Tumor blocks were co-cultured with DRG according to the method of reference<sup>[9]</sup>. Totally, 10 specimens of the esophageal and cardiac carcinoma respectively were used for co-culture. DRG were isolated from E8-10 chick embryo (obtained from Chinese Academy of Agricultural & Science) and seeded into 24-well plate coated with poly-L-lysine 30 min before co-culture. Tumor specimens were cut into 1 mm<sup>3</sup> blocks to co-culture with DRG at a distance of 1.5-2 mm. Each well had one tumor block and one ganglion. Each specimen was co-cultured for 12 wells. They were then incubated at 37 °C with 7.5 % CO<sub>2</sub> for 4hrs to attach. After that, DMEM without any serum was added into the wells. In the control group, DRG were cultured without tumor blocks in the same medium as in the experimental group. There were 10 wells of DRG cultured as control for each case of tumor specimens. The co-cultures were observed under a phase-contrast microscope (IX70, OLYMPUS) 24hrs later and the DRG with processes outgrowth were counted for statistical analysis.

### Statistical analysis

The completely randomized design *t* test was used to analyze for statistical significance. The *P* values less than 0.05 were considered as significantly different.

## RESULTS

### Distribution of neuropeptide-immunoreactive nerve fibers in tumor and their relationship with tumor cells

Under light microscope, substantial amounts of many kinds of neuropeptide-immunoreactive nerve bundles and scattered nerve fibers were observed in both esophageal and cardiac carcinomas. Many of them were distributed around blood vessels and in the connective tissues. But more importantly, a lot of scattered nerve fibers were distributed among tumor cells

(Figures 1-6). In this study, only those areas with neuropeptide immunoreactive nerve fibers scattered among tumor cells were specified as positive. Distribution of positive nerve fibers in tumor was scored as 0 to 3+. The tumor sections with >2/3 positive area were scored as +++, 1/3-2/3 positive area were ++ and <1/3 positive area were + (Table 2). The frequency of neuropeptides-containing nerve fibers varied among individual tumor specimens. In some cases, the positive nerve fibers were numerous around blood vessels and in the parenchyma, while in others there were only a few fibers running along blood vessels. Relatively, in tumors with good differentiation, the frequency of positive nerve fibers was a little higher than in those with poor differentiation. To a great extent, the distribution pattern and frequency were similar in esophageal and cardiac carcinomas. GAL- and NPY-immunoreactive nerve fibers were numerous in the parenchyma in the both carcinomas, whereas CCK- and SOM-immunoreactive nerve fibers were scarce. No NT-immunoreactive nerve fibers could be detected. In the sections of the negative control group, no immunoreactive nerve fibers were observed.

**Table 2** Distribution of neuropeptide-immunoreactive nerve fibers in tumors

Antibodies	Cardiac cancer (n=13)			Esophageal cancer (n=16)		
	+++	++	+	+++	++	+
CGRP	0	2	2	0	2	2
GAL	2	6	4	3	7	4
SP	1	5	1	2	5	5
NT	0	0	0	0	0	0
SOM	0	3	3	0	3	6
CCK	0	0	1	0	0	3
L-ENK	1	3	1	0	3	7
Dyn	2	3	5	0	8	1
NPY	2	5	3	1	6	6
M-ENK	3	3	2	0	5	7

The scattered nerve fibers were mainly derived from either the nerve bundles or the connective tissues around (Figure 1). Wherever they came from, they had a common distribution pattern of extending toward the inside of the parenchyma of tumors. Most of the nerve fibers had varicosities aligned on them like beads (Figures 2, 5). Nerve fibers and their varicosities waved their way among tumor cells. Sometimes they were found entering into tumor convergently from the connective tissue around (Figures 3, 4), or extending fine branches into the nest formed by carcinoma cells (Figure 6). They contacted with tumor cells closely; some of them even encircled tumor cells (Figure 2).

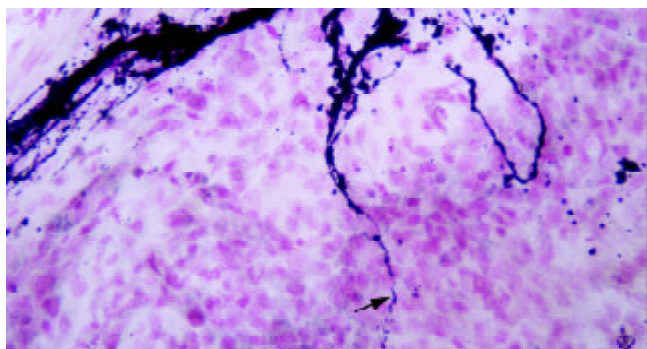
### Outgrowth of processes of DRG in co-cultured group

After incubation for 24-48hr, most of the DRG in the co-culture group extended their processes longer than their diameter (Table 3, Figure 7). Interestingly, the processes were distributed around DRG asymmetrically (Figure 7). On the sides adjacent to tumor blocks, they were dense and long, whereas on the opposite sides, they were sparse and short, or no processes at all. In the control group, no DRG extended their processes.

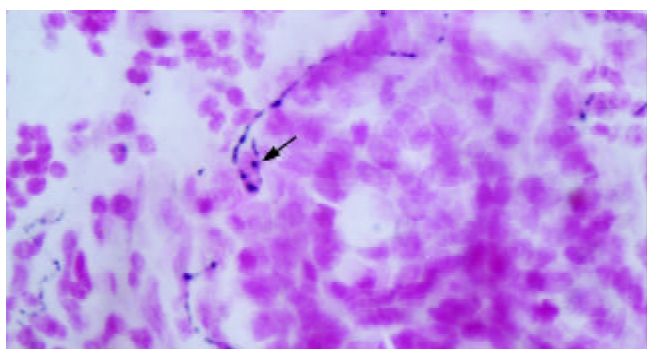
**Table 3** Outgrowth of processes of DRG co-cultured with tumor blocks

Tumor group	DRG with processes (n)	DRG without processes (n)	Rate of DRG with processes (x $\pm$ s)
Esophageal	49	28	0.63 $\pm$ 0.10 <sup>a</sup>
Cardiac	60	29	0.67 $\pm$ 0.09 <sup>a</sup>
Control	0	63	0

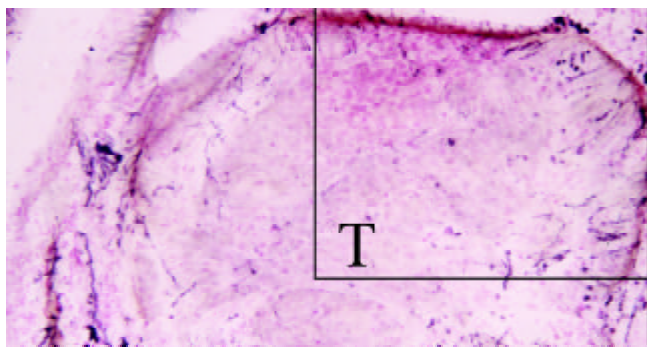
<sup>a</sup>*P*<0.0001 vs the control group



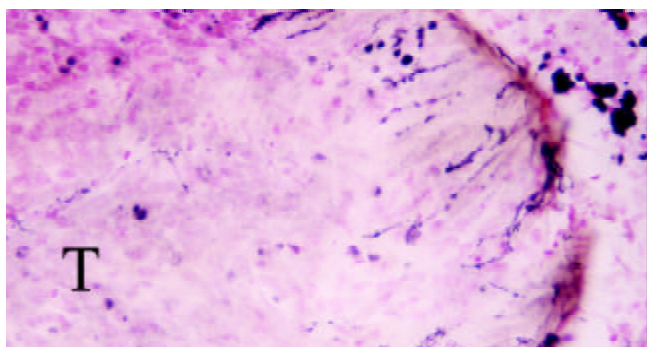
**Figure 1** GAL-immunostaining of esophageal carcinoma. Scattered nerve fibers (arrows) branched almost vertically from nerve bundles in connective tissue and entered into tumor parenchyma  $\times 250$ .



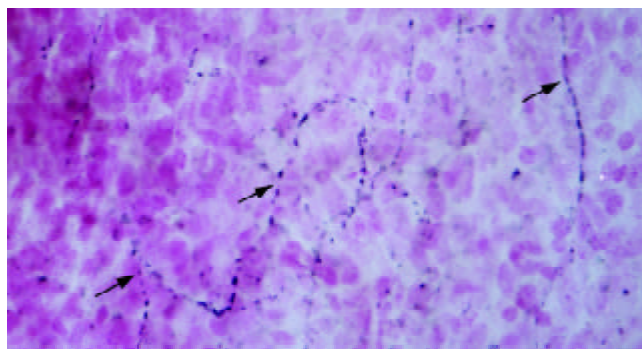
**Figure 2** L-ENK-immunoreactive nerve fibers in esophageal carcinoma. The fine nerve fiber (arrow) with varicosities on it encircled a tumor cell and contacted with it closely  $\times 500$ .



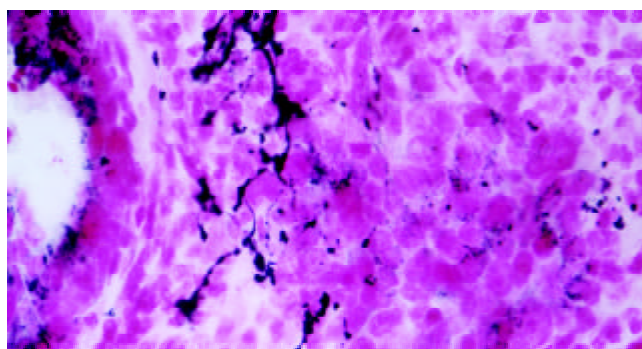
**Figure 3** GAL-immunostaining of esophageal carcinoma. Many nerve fibers entered into a nestlike cancerous tissue (T) convergently from collective tissue around it. The structure on the top right corner was amplified in Figure 4  $\times 125$ .



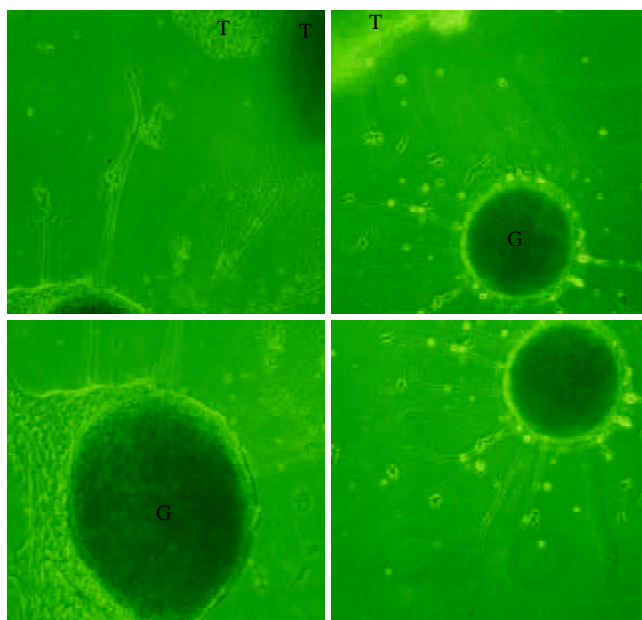
**Figure 4** Higher magnification of the part on the top right in Fig.3. GAL-immunoreactive nerve fibers entered into esophageal carcinoma from peripheral areas  $\times 250$ .



**Figure 5** Section of Cardiac adenocarcinoma immunostained for GAL. Many immunoreactive nerve fibers with beads-like varicosities distributed in the parenchyma of tumor  $\times 500$ .



**Figure 6** GAL-immunoreactive nerve fibers extended into nestlike cancerous tissue formed by cardiac adenocarcinoma cells. The scattered nerve fibers contacted with tumor cells closely  $\times 500$ .



**Figure 7** Montage photographs showing the effects of esophageal (a) and cardiac (b) carcinoma tissue block (T) on co-cultured DRG (G). On the side adjacent to tumor, DRG extending long and dense processes whereas on the opposite side, the processes are sparse and short (b), or no processes at all (a)  $\times 125$ .

## DISCUSSION

Previous studies<sup>[10-12]</sup> have shown that the esophagus is richly supplied with neuropeptidergic nerves. In the present study, the neuropeptide-immunoreactive nerves fibers in the

esophageal and cardiac carcinomas seem to have roughly the same topographic distribution as those in the normal digestive tract in that GAL-, NPY-, ENK- and SP-containing nerves fibers predominate in esophagus and stomach, whereas SOM-, NT-containing nerves fibers predominate in intestine<sup>[13]</sup>. These neuropeptides have very important functions<sup>[14-16]</sup> on the physiological activities of digestive system such as the motility, secretion, etc. Recently, there are more and more evidences showing that neuropeptides can also affect the growth and differentiation of tumors<sup>[17-20]</sup>. But since there are endocrine cells in gastrointestinal wall, it is difficult to distinguish if the neuropeptides come from endocrine cells or nerve terminals<sup>[21-22]</sup>. Therefore, to some extent, the findings of scattered neuropeptides-containing nerve fibers in tumor will help us understand the mechanisms of neuropeptides affecting tumors.

On the other hand, the findings about the distributions of nerve fibers in esophageal and cardiac carcinomas and their close relationship with tumor cells provided a new viewpoint about the innervation of tumors. In a previous report<sup>[2]</sup>, the single adrenergic and NPY-containing nerve fibers were found distributed in the parenchyma of human parathyroid adenomas, but most of them were located in the perivascular space. And no detailed descriptions of their relationship with tumor cells are available. With regard to the perineural invasion by carcinomas<sup>[3,4]</sup>, it was usually considered to be one of the important routes of tumor dissemination and could not be called innervation. In 2001, some German researchers<sup>[23]</sup> observed nerve fibers within an adenoma of the ciliary body epithelium of the eye. Under a transmission electron microscope, a small number of fine unmyelinated nerve fibers were found containing clear and dense core vesicles in tumors. Recently, they examined the exophytic tumors of the urinary bladder and human choroidal melanoma<sup>[24,25]</sup>, and also found irregularly distributed fine nerve strands comprised of axons; hence they concluded that tumors may be innervated. In this regard, they happened to have the same view with us. In this study, we found many scattered nerve fibers not only around blood vessels, but also in the parenchyma of tumors and among tumor cells. Although we did not find specific structure of synapse under electron microscope, these scattered nerve fibers with their beads-like varicosities attached with tumor cells so closely that we can reasonably deduce that they must have important effects on tumor cells, because varicosities are considered containing vesicles and can form synapse-like structures, and will release neuropeptides or other neurotransmitters in their vesicles when stimulated properly. These results suggest that esophageal and cardiac carcinomas may be innervated by peptidergic nerve fibers. Together with previous studies, we suggest that it is necessary to investigate the validity of the old theory about the innervation of tumor.

Why do we get the results that the previously published data did not? We think we have the following advantages: (1) we used frozen sections to facilitate the preservation of the antigen; (2) we used immunohistochemical ABC method to examine the immunoreactive nerve fibers, which is more sensitive than old methods. (3) Neuropeptides-containing nerve fibers are widely distributed in digestive tract from esophagus to the anal sphincter, which provide the possibility for nerve fibers to grow into tumors.

In order to understand how the nerve fibers grow into tumors, we performed co-culture experiment to investigate if tumors had the ability to induce neurons to extend their processes. Our results affirmed this possibility. The neurons of DRG extended processes and the processes on the side adjacent to tumor blocks were dense and long, suggesting that the esophageal and cardiac carcinoma tissues may secrete neurotrophic factors, which are important for the extending of neuronal processes. The SDS electrophoresis (data not shown)

of the tumor-conditioned medium confirmed this assumption and demonstrated that one of them was NGF. Many kinds of tumors have been found secrete NGF, BDNF, NT-3, etc.<sup>[26-28]</sup>. NGF high-affinity receptor TrkA was also found to exist in esophageal carcinoma<sup>[29]</sup>. Therefore, it is possible that these neurotrophic factors in esophageal and cardiac carcinoma tissues act on the neurons in enteric nervous system and induce their fibers to grow into tumors. Or they may exert their neurotrophic functions by helping the injured nerve trunks in tumors regenerate and sprout new nerve fibers. These nerve fibers would extend along with the direction of the diffusion of the neurotrophic factors<sup>[30]</sup> and therefore enter into the tumors.

The functional role of these neuropeptide-containing nerve fibers in esophageal and cardiac carcinoma is not clear. The morphological observation indicated that nerve fibers and their varicosities were in close contact with the tumor cells. This would suggest a role of nerve terminals in the regulation of tumor growth and/or differentiation, which is consistent with the function of neuropeptides on tumors. This might partly explain why mental factors could affect the tumorigenesis and tumor development.

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