Gene expression of vasoactive intestinal peptide receptors in human lung cancer

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Abstract. Despite significant improvement in the diagnosis and treatment of various human carcinomas, the 5-year survival rate for lung cancer remains below 20%. Vasoactive intestinal peptide (VIP) is an important neuropeptide in the control of lung physiology, and exerts its functions mainly through two receptor subtypes, VPAC1 and VPAC2. Receptors for VPAC1 and VPAC2 are present in human lung cancer cells, but very limited information exists about the mRNA expression of these VIP receptor subtypes in lung cancer specimens. The aim of the present study was to investigate by RT-PCR the mRNA expression of the VPAC1 and VPAC2 receptors in surgical specimens of 43 human lung cancer specimens and 7 normal lung samples. mRNA expression of the VPAC1 receptor was detected in 51% of the tumor specimens, while the incidence of mRNA expression for VPAC2 was 46%. Twenty-one percent of the tumor samples expressed only the VPAC1 receptor and 16% displayed only the VPAC2 receptor, while 13 samples (30%) expressed neither subtype. Thirteen cancer tissue specimens (30%), expressed both of these VIP receptor subtypes. Three normal lung tissue specimens also displayed gene expression for VPAC1 and/or VPAC2 receptors. Our results support the additional investigation of the role of VIP and its receptors in human lung cancer and suggest a further development of VIP analogs for therapeutic and imaging purposes in this malignancy.

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Introduction

Lung cancer is a major health problem worldwide with about 1.4 million deaths per year (1). While great progress has been made in the diagnosis and therapy of other malignancies, the estimated number of lung cancer cases has increased by 27% since 2000 (1,2) and the 5-year survival of patients with lung cancer is only 6-18% (3). During the last two decades, the presence of receptors for different peptide hormones, including the vasoactive intestinal peptide (VIP), has been shown in human tumor tissues (4,5). Based on these findings, radiolabeled and cytotoxic peptide hormone analogs which can be targeted to their specific receptors, have been developed for tumor localization and therapy of these malignancies (4,5).

VIP is synthesized from a 170 amino acid precursor protein which is metabolized to a pleiotropic 28 amino acid neuropeptide with a relatively high expression in various normal and malignant tissues (6,7). The effects of VIP are mediated through interactions with three receptors, VPAC1, VPAC2 and PAC1, although PAC1 receptors have a much lower binding affinity (6,7). VIP receptors are mainly coupled to adenylyl cyclase activation through the Gαs protein and induce a cyclic adenosine monophosphate (cAMP) increase. Elevated cAMP levels lead to protein kinase-A (PKA) activation and phosphorylation of the cAMP response element-binding protein (CREB), and eventually regulate nuclear oncogenes (8). In addition, activated VPAC receptors stimulate the production of intracellular calcium (9) and modulate the activity of phospholipase D (PLD) (10). Although VIP is distributed in the entire body, VPAC1 and VPAC2 receptors exhibit somewhat different expression patterns. VPAC1 receptors can be found in many tissues such as the liver, kidney, prostate, breast, spleen, lung, gastrointestinal tract and lymph nodes (11). VPAC2 receptors are mainly present in smooth muscle cells of different organs and blood vessels and in different areas of the central nervous system (12). In the airways, VIP has been implicated in the relaxation of nonvascular and vascular smooth muscle, as well as in the stimulation of mucus secretion, and vasodilatation (13).

The presence of specific VIP receptors has also been demonstrated in human cancer cells including small cell lung

cancer (SCLC) and non-small cell lung cancer (NSCLC) cells and by different detection methods in specimens of primary and metastatic lesions of various human tumors (5-7,11,12). Because of the frequent expression of VIP receptors in various cancers, several VIP analogs have been evaluated for tumor therapy and imaging. A VIP antagonist (VIPhybrid) inhibited the proliferation of NSCLC *in vitro* and decreased the growth of NSCLC tumors xenografted into nude mice (14). This attenuation of tumor proliferation was proven to be receptor-mediated as the VIPhybrid inhibited the specific ^{125}I-labeled VIP binding to NSCLC cell lines (14). It was also demonstrated that the VIPhybrid potentiates the cytotoxicity of chemotherapeutic agents in cancer cell lines (15). Vasoactive intestinal peptide/growth hormone-releasing hormone (VIP/GHRH) antagonists, such as JV-1-52 and JV-1-53 constructed by our group, were also able to inhibit the growth of androgen-independent prostate cancers by abrogating the autocrine/paracrine mitogenic stimuli of VIP (16). In prostate cancers, a probe specific for a ^{64}Cu-labeled receptor for PET imaging delineated xenografts and cases of occult prostate carcinoma that were not detectable with ^{18}F-FDG (17). It was shown that the VPAC1 receptor density in human lung cancer cell lines is unusually high being comparable to the density of tyrosine kinase receptors (6,18). Because of this high density of VPAC1 receptors in cancer cells, it may be possible to utilize VPAC1 receptors to image tumors.

mRNA for VIP could be detected in several lung cancer cell lines (19), but the presence of the two main VIP receptors, VPAC1 and VPAC2, has been studied in only a limited number of lung cancer specimens. Moreover, the findings on of the expression of VPAC1 and VPAC2 receptors in surgical specimens of NSCLC, SCLC and normal lung tissues are incomplete and conflicting (7,20,21). To address this issue, in the present study we investigated the incidence and expression pattern of VPAC1 and VPAC2 receptor genes in 43 malignant and 7 normal human lung tissue samples by a highly sensitive RT-PCR method.

Materials and methods

Tissue samples. Specimens of human lung carcinomas from 43 patients (19 female and 24 male; range, 39-73 years of age) were collected by the Department of Pulmonology, University of Debrecen, Debrecen, Hungary. Seven normal lung tissue specimens were obtained from surgery on lung cancer patients and consisted of histologically confirmed normal lung tissue portions adjacent to the major lung cancer focus. After surgical removal, all samples were flash frozen and stored at -70˚C. Histopathological examinations of each specimen were carried out at the Department of Pathology, University of Debrecen. The collection of human tissue specimens and the experimental protocol were approved for the current study by the Institutional Ethics Committee of University of Debrecen and the patients gave informed consent. The main clinico-pathological characteristics of patients with lung carcinoma are summarized in Table I.

RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR). Total-RNA was extracted from 20-30 mg of homogenized tissue using the RNeasy mini kit (Qiagen, Germany) following the manufacturer's instructions but including an extensive DNA digestion step by DNase. RNA concentration and purity were determined using a NanoDrop ND-1000 UV spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Subsequently, 250 ng of total-RNA was reverse transcribed using oligo(dt) primers and Moloney murine leukemia virus reverse transcriptase (Promega Co., Madison, WI, USA) according to the manufacturer's instructions. PCR was performed applying 1 μl cDNA, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 200 μM of each dNTP, 1 U Taq polymerase and 1X PCR-buffer (all reagents from Invitrogen, Carlsbad, CA, USA) in a final volume of 25 μl. Oligonucleotide primers were designed using the Primer3 software (22) in different exons to help prevent amplification of genomic DNA. The main characteristics of the primers used are summarized in Table II. Initial denaturation for 3 min at 94˚C was followed by amplification of 30 cycles (β-actin) or 40 cycles (VPAC1 and VPAC2 receptors) of 45 sec at 94˚C for template denaturation, 30 sec at 58˚C (VPAC1 and VPAC2 receptors) or 60˚C (β-actin) for annealing and 90 sec at 72˚C for extension, with a final elongation step at 72˚C for 10 min. PCR products were separated electrophoretically on 1.5% agarose gel stained with ethidium bromide and visualized under UV light.

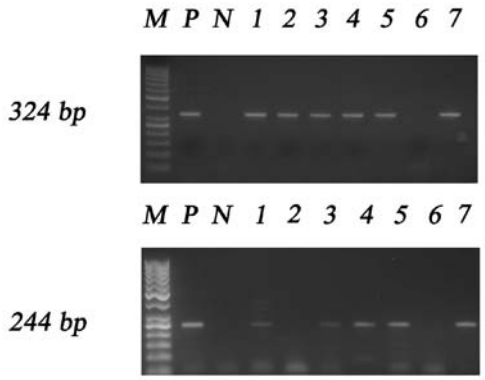

Figure 1. Representative RT-PCR analysis of mRNA for VPAC1 receptors (top panel, 324 bp) and VPAC2 receptors (bottom panel, 244 bp) in human lung cancer specimens. PCR products were separated electrophoretically on 1.5% agarose gel and stained with ethidium bromide. Lane M, molecular DNA marker (50 bp); lane P, positive control (VPAC1 and VPAC2 receptor positive human kidney cancer specimen); lane N, no template control; lanes 1-7, human lung cancer samples.

Results

The clinicopathological data of the specimens investigated and the results of RT-PCR analyses are shown in Table I and Table III. We were able to isolate RNA in an appropriate quality and quantity from 43 tumor samples and 7 normal lung tissues. For all samples, a β-actin-specific RT-PCR was carried out to confirm the efficiency of the reverse transcription. Normal and cancerous lung tissues (including 2 SCLC, 36 NSCLC, 1 carcinoid tumor and 4 lung metastases) were analyzed by RT-PCR for the presence of VPAC1 and VPAC2 receptors. As shown in Fig. 1, the bands corresponding to the amplification products obtained with the specific primer pairs showed the expected sizes of 324 bp for VPAC1 receptor and 244 bp for VPAC2 receptor.

Table I. Clinicopathological findings and mRNA expression pattern of VPAC1 and VPAC2 receptors (VPAC1-R and VPAC2-R) in human lung cancer specimens.

Case no.[a]	Age at diagnosis (years)	Gender	TNM	Tumor type	Diagnosis	VPAC1-R	VPAC2-R
1	50	F	T1N0Mx	SCLC	Microcellular cc	+	+
2	68	F	T1NxMx	SCLC	Microcellular cc	-	-
3	55	F	T1N0Mx	NSCLC	Adenocarcinoma	+	+
4	58	F	T1N0Mx	NSCLC	Adenocarcinoma	+	+
5	63	F	T1N0Mx	NSCLC	Adenocarcinoma	+	+
6	60	M	T1N1Mx	NSCLC	Adenocarcinoma	+	+
7	55	M	T2aN0Mx	NSCLC	Adenocarcinoma	+	+
8	59	M	T2aN2Mx	NSCLC	Adenocarcinoma	+	+
9	68	M	T2N0Mx	NSCLC	Adenocarcinoma	+	+
10	53	F	T2N0Mx	NSCLC	Adenocarcinoma	+	+
11	52	F	T2N0Mx	NSCLC	Adenocarcinoma	+	+
12	53	M	T2N2M1	NSCLC	Large-cell anaplastic cc	+	+
13	73	F	T2N2Mx	NSCLC	Adenosquamous cc	+	+
14	52	M	T3aN0Mx	NSCLC	Adenocarcinoma	+	+
15	55	F	T1N1Mx	NSCLC	Planocellular cc	+	-
16	44	F	T2N0M1	NSCLC	Adenocarcinoma	+	-
17	54	M	T2N0Mx	NSCLC	Papillary adenocarcinoma	+	-
18	51	F	T2N0Mx	NSCLC	Clarocellular cc	+	-
19	73	F	T2N2Mx	NSCLC	Adenosquamous cc	+	-
20	54	M	T3N2Mx	NSCLC	Adenopapillary cc	+	-
21	52	M	T1N0Mx	NSCLC	Adenocarcinoma	-	+
22	58	F	T1N0Mx	NSCLC	Bronchoalveolar adenocarcinoma	-	+
23	72	M	T1NxMx	NSCLC	Planocellular cc	-	+
24	68	M	T2N0Mx	NSCLC	Adenocarcinoma	-	+
25	56	F	T2N1Mx	NSCLC	Planocellular cc	-	+
26	56	F	T2N1Mx	NSCLC	Planocellular cc	-	+
27	54	M	T1N0Mx	NSCLC	Planocellular cc	-	-
28	56	M	T1N0Mx	NSCLC	Adenocarcinoma	-	-
29	53	M	T1N1Mx	NSCLC	Adenocarcinoma	-	-
30	65	M	T1N2Mx	NSCLC	Adenocarcinoma	-	-
31	62	M	T2N0Mx	NSCLC	Planocellular cc	-	-
32	63	M	T2N0Mx	NSCLC	Planocellular cc	-	-
38	59	M	T2N0Mx	NSCLC	Adenocarcinoma	-	-
33	59	M	T2N1Mx	NSCLC	Adenocarcinoma	-	-
34	59	M	T3N0Mx	NSCLC	Planocellular cc	-	-
35	59	M	T3N0Mx	NSCLC	Planocellular cc	-	-
36	39	M	T3N1M0	NSCLC	Planocellular cc	-	-
37	53	F	T3N1M0	NSCLC	Adenocarcinoma	-	-
39	59	F		Carcinoid		-	+
40	52	F		Metastasis		+	-
41	73	M		Metastasis		+	-
42	54	F		Metastasis		-	-
43	59	M		Metastasis		+	-

[a]Order of samples is based on tumor type and VPAC1 and VPAC2 receptor positivities. cc, carcinoma.

Table II. Main characteristics of the primers used for the detection of VPAC1 and VPAC2 receptors (VPAC1-R and VPAC2-R) and a control housekeeping gene (β-actin).

Primer	Sequence	GenBank ID	Localization	Product size
VPAC1-R				
Sense	5'-atgtgcagatgatcgaggtg-3'	NM_004624.2 GI:15619005	Exon 2	324 bp
Antisense	5'-tgtagccggtcttcacagaa-3'		Exon 5	
VPAC2-R				
Sense	5'-ccagaatgccgatttcatct-3'	NM_003382.3 GI:33188464	Exon 2	244 bp
Antisense	5'-ctgggaacgtctctgaccat-3'		Exon 4	
β-actin				
Sense	5'-ggcatcctcaccctgaagta-3'	NM_001101.2 GI:5016088	Exon 3	172 bp
Antisense	5'-ggggtgttgaaggtctcaaa-3'		Exon 4	

Table III. Expression of mRNA for VPAC1 and VPAC2 receptors (VPAC1-R and VPAC2-R) in normal and cancerous human lung tissue pairs.

		Cancer tissue		Normal tissue	
Case no.	Tumor type	VPAC1-R	VPAC2-R	VPAC1-R	VPAC2-R
17	NSCLC	+	-	-	-
18	NSCLC	+	-	+	-
20	NSCLC	+	-	-	-
24	NSCLC	-	+	+	+
27	NSCLC	-	-	-	-
39	Carcinoid	-	+	-	-
41	Metastasis	+	-	+	-

Expression of mRNA for VPAC1 and VPAC2 receptors in lung cancer tissues. The mRNA for VPAC1 receptors was detected in 51% of the tumor specimens, while the incidence of receptors for VPAC2 was 46%. Twenty-one percent of the tumor samples expressed only the VPAC1 receptor, 16% displayed solely the VPAC2 receptor, and 13 samples (30%) showed neither subtype. In 13 cancer tissues (30%), mRNA for the two VIP receptor subtypes was found (Table I).

In NSCLC samples, VPAC1 and VPAC2 receptors showed a similar expression, with both being present in 50% of the tumors analyzed (Table I). Of the 36 NSCLC specimens, 12 (33%) displayed both VIP receptor subtypes; 6 samples (17%) expressed only VPAC1 and 6 tumors (17%) only VPAC2 receptors. Most NSCLC specimens (10/12) showing a co-expression of both receptors were adenocarcinomas. One of the 2 SCLC samples expressed mRNA for both VIP receptor subtypes but the other specimen displayed neither subtype. (Table I) One carcinoid tumor specimen examined expressed VPAC2 receptors only. Of the 4 lung cancer metastases, 3 displayed mRNA for only VPAC1 receptors and 1 sample showed neither VIP receptor subtype (Table I).

Interestingly, among the 13 tumors which expressed neither VPAC1 nor VPAC2 receptors, 11 were from male patients (Table I). Otherwise, no correlation between age at diagnosis, gender of the patients or tumor staging and VIP receptor mRNA expression could be found.

Expression of mRNA for VPAC1 and VPAC2 receptors in normal lung tissue - lung cancer tissue pairs. For 7 patients, both normal lung and lung cancer tissues were available for molecular biology studies (Table III). Three of the 7 normal lung tissues examined expressed VPAC1 receptors, and only one sample expressed both VPAC1 and VPAC2 receptors. Four non-cancerous lung tissues displayed neither VIP receptor subtype, while their malignant counterparts showed VPAC1 receptor expression in 2 cases, and VPAC2 receptor expression in one case. In the tumor counterpart of the normal sample from the same patient that expressed both types of receptors, mRNA for only VPAC2 receptors could be detected by RT-PCR. The results of RT-PCR in normal lung tissue and lung cancer tissue pairs are summarized in Table III.

Discussion

Accumulating evidence indicates that neuropeptides such as VIP are involved in the mechanisms of progression of various human cancers. VIP is a member of a superfamily of structurally related peptide hormones that includes growth hormone-releasing hormone (GHRH), pituitary adenylate

cyclase-activating polypeptide (PACAP), secretin and glucagon (23). VIP binds to two types of VIP receptors, previously called VIP1 and VIP2 receptors or to PACAP type-2 receptors, which also have a high affinity for PACAP. Therefore, these VIP receptors were recently named VPAC1 and VPAC2 receptors (24). These receptors with different tissue distribution and pharmacological properties are distinct from the specific highly selective PACAP type-1 receptors that recognize VIP with a low binding affinity (25). VIP and PACAP bind with high affinity to their respective receptors and are also able to crossreact to various degrees, in general with reduced binding affinity, with the receptors of the other members of this superfamily because of the structural similarity of the peptides and their receptors (26). The two subtypes of receptors for VIP, VPAC1 and VPAC2, are abundant in many types of human cancers (5-7,11,12,20,27).

In the human lung, the presence of VPAC receptors has been demonstrated by RT-PCR, immunoblotting and immunohistochemical staining (28,29). There is also growing evidence of the importance of neuropeptides in the pathophysiology and progression of human lung cancer. Previous studies have shown that VIP is an important regulatory peptide and may function as an autocrine growth factor in SCLC and NSCLC (6,14,30,31). The mRNA expression of VIP and its immunoreactivity has been demonstrated in several lung cancer cell lines (19). VIP binding sites have also been described in human lung cancer cell lines, both in SCLC (6,18,32) and NSCLC cells (33,34).

Busto *et al* reported the expression of different subtypes of VPAC receptors in human lung tumor samples by using specific primers for RT-PCR and specific antibodies for immunobloting and immunohistochemistry studies (21). An earlier study detected predominantly VPAC1 receptors in NSCLC but investigated only 11 lung carcinoma samples (7). Autoradiography studies have also shown the presence of VIP binding sites in 75% of SCLC and NSCLC specimens (20).

To further investigate the expression of VPAC receptors in human lung cancer and normal lung tissue specimens in a large cohort of patients, we determined the expression of mRNA for VPAC1 and VPAC2 receptor subtypes in surgical specimens of 43 human lung cancers and 7 normal lung tissue specimens using a highly specific RT-PCR technique. Our results indicate that 51% of the tumor specimens expressed VPAC1 receptor mRNA, while the incidence of VPAC2 receptor mRNA expression was 46%. Twenty-one percent of the tumor samples expressed only the VPAC1 receptor and 16% only the VPAC2 receptor, while 30% showed neither receptor subtype. mRNA co-expression of the two VIP receptor subtypes was found in 30% of human lung cancers. These findings are in agreement with the results of Busto *et al* (21). In our study, 33% of the 36 NSCLC specimens displayed both VIP receptor subtypes; 17% expressed only VPAC1 and 17% only VPAC2 receptors. It is important to note that the study of Reubi *et al* (7) on only 11 NSCLC specimens using radioligand competition assays did not detect the presence of VPAC2 receptors. The discrepancy between the present findings and the previous report (7) may be explained by the use of different detection methods. One carcinoid tumor specimen examined expressed only the VPAC2 receptors and 3 out of 4 lung cancer metastases displayed the mRNA solely of the VPAC1 receptor.

In the present study, 3 normal lung specimens showed gene expression for VPAC1 or VPAC2 receptors, although our observations are limited to 7 samples. Our findings support the need for further investigations of the expression of VPAC receptor subtypes in human lung tissues at the mRNA and protein level as well.

It was reported earlier that the density of VPAC1 receptors in human lung cancer cell lines is approximately an order of magnitude greater than that of VPAC2 receptors (6,18,33). Based on the presence of high density VPAC1 receptors in various cancers including SCLC and NSCLC, radiolabeled VIP analogs were used to localize various primary tumors and their metastases (5,6,17,20). However, it remains to be determined if a radiolabeled VIP analog can be useful for the early detection of lung cancer. The presence of receptors for VIP on human cancers also led to the investigation of specific antagonists of VIP, PACAP and VIP/GHRH for the treatment of lung cancers and other malignancies in which VIP may function as an autocrine growth factor (6,14,16,30). It was reported that VIPhybrid (14) and PG 97-269 selective to VPAC1 receptors (35) could be potential anti-tumor agents. The GHRH analogs JV-1-52 and JV-1-53, which have a diminished GHRH antagonistic activity, but show increased antagonistic actions on VPAC1 and VPAC2 receptors have been synthesized (36). JV-1-53 exhibits extremely high VIP antagonistic activity in various biological assays aimed at evaluating its inhibitory effects on the pharmacological actions of VIP, dependent on VPAC1 and VPAC2 receptors (36). It was shown that these and other VIP antagonists significantly inhibited the growth of human experimental NSCLCs, mammary, prostate and pancreatic carcinomas *in vitro* and *in vivo* (6,14,16,30,35,36).

In conclusion, the present study extends previous investigations on the expression of VPAC1 and VPAC2 receptor subtypes in human SCLC and NSCLC. The demonstration of a high incidence of VPAC1 and VPAC2 receptors in human lung cancers stongly supports the need for further investigations on the applications of VIP analogs and their radionuclide derivatives for receptor-based diagnosis and treatment of SCLC and NSCLC.

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