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ORIGINAL ARTICLE

Cathepsin D messenger RNA is downregulated in human lung cancer

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Abstract

Objectives: Lysosomal proteases cathepsins B and D (CB and CD) play a significant part in cancer progression. For many oncological diseases protein expression levels of CB and CD have been investigated and correlations with tumour characteristics revealed. Meanwhile, there is very little information concerning mRNA expression level.

Methods: In the present work, data about mRNA levels of CB and CD in human lung cancer was obtained using reverse transcription followed by real-time polymerase chain reaction.

Results: For the first time CD and CB mRNA in human lung cancer tumours was quantified. It was shown that CB and CD mRNA levels do not correlate with any tumour characteristics. However, in most analysed tumours, expression of CD mRNA was downregulated compared with adjacent normal tissue (p <0.0003).

Conclusions: The data obtained indicate CD mRNA as a potential lung cancer marker.

Keywords: *Cathepsin B; cathepsin D; lung cancer; gene expression; real-time PCR*

Introduction

According to present understanding, cathepsins B and D (CB and CD), lysosomal proteolytic enzymes, play a significant role in malignant tumour progression (reviewed in Podgorski & Sloane 2003, Leto et al. 2004, Liaudet-Coopman et al. 2006). Development of neoplasms of different origin has been shown to be accompanied by heightened secretion of CB and CD (Capony et al. 1989, Sloane et al. 1994, Hazen et al. 2000, Turk et al. 2004). Secreted CB due to both intrinsic proteolytic activity and activation of matrix protease precursors causes extracellular matrix degradation, which evidently assists invasion and dissemination of tumour cells (Keppler et al. 1996, Roshy et al. 2003, Sevenich et al. 2010). The effect of CD on neoplasm progression is linked with its proteolytic activity: CD has an ability to facilitate release of angiogenic factors from the extracellular matrix (Briozzo et al. 1991). In addition, it was shown that the non-active CD precursor acts on cancerous and adjacent normal cells as a mitogenic factor (Fusek & Vetvicka 1994, Glondu et al. 2001, Vashishta et al. 2006, Fusek et al. 2007, Ohri et al. 2008, Masson et al. 2010).

Correlations between CD and CB mRNA and protein expression levels on the one hand, and tumour stage, aggressiveness, probability of disease relapse and post-operative survival on the other hand were reported for stomach, breast, brain, prostate, colon and other types of cancer (Murnane et al. 1991, Nazeer et al. 1992, Schwartz 1995, Cherry et al. 1998, Foekens et al. 1999, Konduri et al. 2001, Levicar et al. 2002, Bossard et al. 2003, Leto et al. 2004, Niedergethmann et al. 2004, Troy et al. 2004, Nomura & Katunuma 2005, Czyzewska et al. 2008, Devetzi et al. 2009). These data allow CB and CD to be

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regarded as potential diagnostic markers of malignant tumours suitable to assay relapse risks (Schwartz 1995, Lah et al. 2000, Wozniak et al. 2002, Bossard et al. 2003, Niedergethmann et al. 2004, Turk et al. 2004, Devetzi et al. 2009).

Expression of cathepsins B and D was also investigated in lung cancer – the most widespread oncological disease, which causes 1.4 million deaths annually (WHO 2009). The level of CB protein in lung tumours was reported to be increased (Sukoh et al. 1994, Werle et al. 1994, 1995, 2000, 2004, Ledakis et al. 1996, Sloman et al. 1996), and correlations with tumour stage, aggressiveness and postoperative survival were revealed in certain cases (Sukoh et al. 1994, Werle et al. 1999, 2000, Fujise et al. 2000, Werle et al. 2000, Cordes et al. 2009). However, the same correlations were not demonstrated in other similar cases (Mori et al. 1997, Werle et al. 1995). The data concerning CD protein content in lung tumours and its correlations with tumour characteristics are also contradictory (Chyczewski et al. 1997, Wang & Zhao 1998, Wozniak et al. 2002, Ruibal et al. 2003, Werle et al. 2004). Subsequently, the data concerning cathepsins B and D expression in lung cancer were obtained on a protein level. However, to our knowledge, there is no information concerning their mRNA. In the present work for the first time quantitative data about CB and CD mRNA levels in human lung cancer were obtained using reverse transcription followed by a real-time polymerase chain reaction (rtPCR) method.

Methods

Collection of tissue samples

Specimens of cancerous tissues and of adjacent tissues without histological pathology (further referred to as 'normal' tissue) were taken from 30 patients with a diagnosis of 'small-cell lung carcinoma' and 'non-small-cell lung carcinoma' (tumour stage I–III) during surgical operations (Table 1). The normal tissue specimens were taken from the edge of resections (distance between tumour and normal tissues was no less than 20 mm). The all patients were under medical supervision in the Blokhin Cancer Research Center (Moscow, Russia) during a period from May 2004 to November 2005. None of these patients received radio- or chemical therapy up to the moment of the investigation. All patients gave written informed consents, and the local Institutional Review Board approved the project.

Each specimen was split into two portions. The first part was frozen in liquid nitrogen immediately for further mRNA isolation. The second portion was sent for histological examination, which was performed after haematoxylin and eosin staining of paraffin sections. Cancer tissue specimens contained more than 70% of malignant cells. In normal tissue specimens there were no malignant cells.

RNA isolation and purification

The total RNA was isolated from samples of cancer and normal tissues with guanidine isocyanate lysis and acid–phenol extraction with subsequent removal of polysaccharide admixtures (Chomczynski & Mackey 1995). Additional purification was performed by RNA precipitation using the RNAeasy Minikit (Qiagen, Valencia, CA, USA). Further treatment with DNaseI (Promega, Madison, WI, USA) was done according to the supplier's recommendation. The RNA samples obtained were characterized electrophoretically in 1% agarose gel. RNA concentration was determined spectrophotometrically.

Double-strand cDNA synthesis

To perform reverse transcription, oligonucleotides AAGCAGTGGTATCAACGCAGAGTACGCrGrGrG and AAGCAGTGGTATCAACGCAGAGTACT$_{30}$VN (V = C, or G or A) (Syntol, Moscow, Russia) were used. For first strand cDNA synthesis, 1 μg of isolated RNA was treated with reverse transcriptase PowerScript (Clontech, Palo Alto, CA, USA) as described by Zhu et al. (2001). The obtained reaction mixture was used for synthesis of a second strand of cDNA and subsequent making of PCR amplification products. Synthesis was performed using Advantage 2 DNA polymerase (Clontech) and primer AAGCAGTGGTATCAACGCAGAGT in the following conditions: 95°C for 1.5 min, then 95°C for 20 s, 65°C for 20 s and 72°C for 3 min for the required number of cycles. To obtain equal amounts of amplification products, the number of cycles carried out varied depending on the sample, but did not exceed 17. In most cases 15 cycles were carried out.

Real-time PCR

To perform rtPCR primers and probes of TaqMan Gene Expression Assays system (Applied Biosystems, Foster City, CA, USA) were used: Hs00157201_ml for the gene of cathepsin D (*CTSD*) and Hs00157194_ml for the gene of cathepsin B (*CTSB*). For the reference gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), TaqMan Pre-Developed Assay Reagent GAPDH 20x (Applied Biosystems) was used. PCR was conducted using a Chromo4 Dyad Disciple cycler (BioRad, Hercules, CA, USA) in line with the supplier's recommendations according to the following program: 50°C for 2 min, 95°C for 10 min, then (95°C for 15 s, 60°C for 60 s) for 45 cycles; the reaction mixture volume was 20 μl. Every sample was tested at least twice in duplicates. The threshold cycle was defined using Opticon Monitor 3 software (BioRad).

Table 1. Characteristics of tumour specimens and levels of gene expression.

Specimen number	Type of carcinoma	Localization	Stage	Keratinization	TNM-classification			Normalized gene expression level (Ratio (*CTS*/*GAPDH*))			
								Cathepsin B		Cathepsin D	
					T	N	M	Normal	Tumour	Normal	Tumour
1	GL-SCC	P	III	-	3	1	0	$(5.7 \pm 0.7)\times10^{-01}$	$(5.0 \pm 1.1)\times10^{-02}$	$(1.4 \pm 0.2)\times10^{-03}$	$(1.0 \pm 0.4)\times10^{-01}$
2	SCC	P	III	N	2	0	0	$(3.0 \pm 0.4)\times10^{-01}$	$(5.9 \pm 0.9)\times10^{-02}$	$(2.6 \pm 1.1)\times10^{-03}$	$(1.1 \pm 1.7)\times10^{-04}$
3	SCC	C	I	N	1	0	0	$(2.2 \pm 0.3)\times10^{-01}$	$(7.7 \pm 0.7)\times10^{-02}$	$(1.1 \pm 0.6)\times10^{-03}$	$(5.0 \pm 3.8)\times10^{-05}$
4	SCC	C	II	Y	3	0	0	$(8.1 \pm 0.8)\times10^{-01}$	$(1.3 \pm 0.4)\times10^{-01}$	$(1.9 \pm 0.8)\times10^{-03}$	$(6.8 \pm 0.5)\times10^{-06}$
5	SCC	C	II	N	3	0	0	$(2.0 \pm 0.3)\times10^{-01}$	$(1.7 \pm 0.3)\times10^{-01}$	$(5.5 \pm 3.0)\times10^{-04}$	$(7.8 \pm 2.6)\times10^{-05}$
6	SCC	C	II	Y	3	0	0	$(5.8 \pm 0.5)\times10^{-01}$	2.5 ± 0.3	$(1.7 \pm 1.1)\times10^{-03}$	$(6.1 \pm 1.8)\times10^{-03}$
7	SCC	C	I	N	1	0	0	$(2.0 \pm 0.2)\times10^{-01}$	$(9.1 \pm 1.3)\times10^{-02}$	$(1.2 \pm 0.2)\times10^{-03}$	$(4.5 \pm 4.2)\times10^{-04}$
8	SCC	C	II	Y	3	0	0	$(5.9 \pm 0.4)\times10^{-01}$	$(3.0 \pm 0.5)\times10^{-01}$	$(1.0 \pm 0.7)\times10^{-03}$	$(0.9 \pm 1.1)\times10^{-04}$
9	SCC	C	III	Y	3	2	0	$(9.0 \pm 1.8)\times10^{-02}$	$(6.2 \pm 1.6)\times10^{-02}$	$(4.2 \pm 2.8)\times10^{-04}$	$(3.1 \pm 3.3)\times10^{-04}$
10	SCC	C	II	N	3	0	0	$(4.3 \pm 0.9)\times10^{-01}$	1.5 ± 0.4	$(0.9 \pm 1.0)\times10^{-03}$	$(4.3 \pm 1.0)\times10^{-04}$
11	SCC	C	II	N	3	0	0	1.2 ± 0.2	$(1.6 \pm 0.2)\times10^{-01}$	$(5.4 \pm 2.9)\times10^{-03}$	$(4.5 \pm 2.4)\times10^{-04}$
12	SCC	P	III	Y	3	1	0	$(1.6 \pm 0.2)\times10^{-01}$	$(2.9 \pm 0.5)\times10^{-01}$	$(8.2 \pm 3.9)\times10^{-04}$	$(4.7 \pm 2.5)\times10^{-05}$
13	ADCA	P	II	-	3	0	0	$(1.9 \pm 0.6)\times10^{-01}$	$(1.3 \pm 0.1)\times10^{-01}$	$(4.7 \pm 5.0)\times10^{-04}$	$(2.3 \pm 0.5)\times10^{-04}$
14	ADCA	P	II	-	2	1	0	$(1.3 \pm 0.5)\times10^{-01}$	$(1.3 \pm 0.2)\times10^{-01}$	$(1.7 \pm 0.2)\times10^{-05}$	$(3.3 \pm 0.8)\times10^{-06}$
15	ADCA	P	II	-	3	0	0	$(1.8 \pm 0.9)\times10^{-01}$	$(1.5 \pm 0.4)\times10^{-01}$	$(2.1 \pm 0.5)\times10^{-05}$	$(5.1 \pm 0.5)\times10^{-06}$
16	SCC	P	II	Y	3	0	0	$(7.7 \pm 3.4)\times10^{-01}$	3.0 ± 0.9	$(1.1 \pm 0.9)\times10^{-04}$	$(6.6 \pm 0.9)\times10^{-04}$
17	SCC	P	II	Y	3	0	0	$(4.4 \pm 1.6)\times10^{-01}$	$(2.4 \pm 0.7)\times10^{-01}$	$(9.5 \pm 2.3)\times10^{-04}$	$(4.4 \pm 1.7)\times10^{-04}$
18	SCC	P	III	Y	2	2	0	$(2.8 \pm 0.8)\times10^{-01}$	$(2.9 \pm 1.1)\times10^{-01}$	$(3.4 \pm 1.2)\times10^{-04}$	$(1.7 \pm 0.6)\times10^{-04}$
19	SCC	P	I	N	1	0	0	$(4.5 \pm 1.3)\times10^{-01}$	$(2.6 \pm 1.1)\times10^{-01}$	$(1.9 \pm 0.6)\times10^{-03}$	$(1.1 \pm 0.5)\times10^{-04}$
20	SCC	P	II	N	3	0	0	$(2.1 \pm 0.1)\times10^{-01}$	$(5.0 \pm 0.8)\times10^{-01}$	$(1.1 \pm 0.8)\times10^{-03}$	$(3.1 \pm 2.4)\times10^{-04}$
21	ADCA	P	II	-	3	0	0	$(3.7 \pm 1.0)\times10^{-01}$	$(4.0 \pm 1.2)\times10^{-01}$	$(1.4 \pm 0.8)\times10^{-03}$	$(7.1 \pm 3.2)\times10^{-05}$
22	SCC	C	II	N	3	0	0	$(3.0 \pm 1.1)\times10^{-01}$	$(7.2 \pm 3.3)\times10^{-01}$	$(4.4 \pm 0.1)\times10^{-02}$	$(1.9 \pm 0.1)\times10^{-01}$
23	SCC	C	II	Y	3	0	0	$(1.2 \pm 0.5)\times10^{-01}$	$(2.6 \pm 1.0)\times10^{-01}$	$(4.2 \pm 1.4)\times10^{-02}$	$(1.2 \pm 0.2)\times10^{-02}$
24	SCC	C	I	N	1	0	0	$(1.8 \pm 0.2)\times10^{-01}$	$(5.3 \pm 0.6)\times10^{-01}$	$(2.4 \pm 0.3)\times10^{-02}$	$(1.7 \pm 0.4)\times10^{-02}$
25	SCC	C	II	N	2	1	0	$(6.2 \pm 1.2)\times10^{-01}$	$(5.6 \pm 0.9)\times10^{-02}$	$(1.4 \pm 0.7)\times10^{-03}$	$(4.2 \pm 2.9)\times10^{-05}$
26	SCC	C	I	Y	1	0	0	$(6.7 \pm 1.8)\times10^{-01}$	$(7.2 \pm 1.0)\times10^{-02}$	$(1.9 \pm 0.4)\times10^{-03}$	$(1.6 \pm 0.4)\times10^{-04}$
27	SCC	C	III	Y	3	1	0	2.7 ± 0.2	$(7.6 \pm 1.1\times)10^{-01}$	$(2.3 \pm 0.6)\times10^{-03}$	$(4.5 \pm 5.7)\times10^{-05}$
28	SCC	C	III	N	3	1	0	$(5.3 \pm 1.3)\times10^{-01}$	$(1.1 \pm 0.2)\times10^{-01}$	$(1.5 \pm 1.0)\times10^{-03}$	$(1.7 \pm 0.2)\times10^{-05}$
29	SCLC	P	III	-	-	-	-	$(4.0 \pm 0.3)\times10^{-01}$	$(1.6 \pm 0.2)\times10^{-01}$	$(8.8 \pm 0.9)\times10^{-04}$	$(2.8 \pm 3.5)\times10^{-04}$
30	SCC	C	II	N	3	0	0	$(7.6 \pm 1.1)\times10^{-02}$	$(1.5 \pm 0.2)\times10^{-01}$	$(7.5 \pm 6.0)\times10^{-04}$	$(1.8 \pm 2.1)\times10^{-04}$

SCC, squamous cell lung carcinoma; GL-SCC, glandular–squamous cell lung carcinoma; ADCA, adenocarcinoma; SCLC, small-cell lung carcinoma; P, peripheral tumour localization; C, central tumour localization; Y, tumour with keratinization; N, tumour without keratinization; -, no data.

Experimental data processing

The experimental data obtained for *CTSD* and *CTSB* were normalized to the *GAPDH* mRNA expression levels using the formula: $\text{Ratio}(CTS/GAPDH) = 2^{C_T(GAPDH)-C_T(CTS)}$, and then averaged (Table 1). Confidence intervals were calculated with 95% probability level. Analysis of samples 1, 2, 4, 13 and 27 failed to detect *CTSD* mRNA in tumour tissue in one of two independent experiments, therefore the calculations were done using data obtained in a successful experiment. Analysis of samples 14 and 15 failed to detect *CTSD* mRNA in tumour and normal tissues in both independent experiments, so the C_T was set to be equal 42.

Kruskal–Wallis one-way analysis of variance was used to evaluate influence of tumour type, stage and TNM characteristics on *CTSD* and *CTSB* expression level. The Wilcoxon matched-pairs rank-sum test was used to evaluate whether there was a statistically significant difference between quantities of *CTSD* mRNA in tumour and normal tissue. The statistical analysis was performed with Statistica 6.0 software (StatSoft, USA).

Results and Discussion

In the present study, for the first time, mRNA expression levels of *CTSD* and *CTSB* in malignant tissue of the human lung were evaluated (Table 1). According to information available from the literature, the protein expression level of *CTSB* in lung tumours is upregulated compared with normal tissue (Sukoh et al. 1994, Werle et al. 1994, 1995, 1999, 2000, 2004, Sloman et al. 1996, Mori et al. 1997). However in the samples we analysed the *CTSB* mRNA levels varied considerably (Table 1). *CTSB* mRNA level does not correlate with stage and other tumour clinical characteristics, which is in line with some results obtained for protein expression level (Werle et al. 1995, Mori et al. 1997). It may be because of alternative *CTSB* transcripts with higher translation efficiency present in cancer cells (Yan & Sloane 2003). Divergences between mRNA and protein content may also be due to accumulation of membrane-associated (and consequently, more stable) active CB (Almeida et al. 2001).

We found *CTSD* expression in tumours to be considerably lower relative to adjacent normal tissues and this effect was statistically significant ($p < 0.0003$). This fact is unexpected, as in many studies an increased content of cathepsin D in tumour cells and in tumour-infiltrated macrophages compared with cells of normal tissues was observed (Chyczewski et al. 1997, Wang & Zhao 1998, Wozniak et al. 2002, Werle et al. 2004, Domagala et al. 1992, Joensuu et al. 1995). However, similar to our findings, Ruibal et al. reported decreased CD protein level in lung cancer compared with normal tissue (Ruibal et al. 2003). There is no significant difference in level of *CTSD* mRNA between groups with different types, stages and other clinical characteristics of tumours, and this fact matches only a part of the data for protein levels obtained to date (Chyczewski et al. 1997, Wozniak et al. 2002, Ruibal et al. 2003). To date there are insufficient data to explain the discrepancy in cathepsin D mRNA and protein. Some mechanisms similar to that of post-transcriptional regulation of CB expression mentioned above could occur in this case. Thus, our data suggest the key events of regulation of cathepsin D expression occur on a post-transcriptional level.

It was revealed earlier by proteolytic activity measurements, that expression level of CD exceeds that of CB in cancerous lung tissues (Ledakis et al. 1996). However, we found the opposite situation on the mRNA level (Table 1), which also apparently indicates a post-transcriptional regulation of cathepsins B and D expression.

In conclusion, a comparison of our results with those of earlier published studies demonstrates considerable difference between protein and mRNA expression levels of *CTSD* and *CTSB* in human lung cancer tissues. This fact may be an indication of *CTSD* and *CTSB* regulation on a post-transcriptional level. At the same time, cathepsin D mRNA is downregulated in human lung cancer cells. Our findings suggest that cathepsin D mRNA could be a highly informative biomarker that, along with others, might be useful in lung cancer diagnostics.

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Declaration of interest

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