RESEARCH ARTICLE

Expression and Prognostic Roles of TRPV5 and TRPV6 in Non-Small Cell Lung Cancer after Curative Resection

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Abstract

Purpose: We investigated the expression of epithelial Ca^{2+} channel transient receptor potential vanilloid (TRPV) 5 and 6 in non-small-cell lung cancer (NSCLC) and assessed their prognostic role in patients after surgical resection. Materials and Methods: From January 2008 to January 2009, 145 patients who had undergone surgical resection of NSCLCs were enrolled in the study. Patient clinical characteristics were retrospectively reviewed. Fresh tumor samples as well as peritumor tissues were analyzed for TRPV5/6 expression using immune-histochemistry (IHC) and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Patients were grouped based on their TRPV5 and TRPV6 levels in the tumor tissues, followed up after surgery, and statistically analyzed to examine the prognostic roles of TRPV5 and TRPV6 on patients' survival after surgical resection of NSCLCs. Results: Using IHC, among the 145 patients who had undergone surgical resection of NSCLCs, strong protein expression (grade≥2) of TRPV5 and TRPV6 was observed in a lower percentage of primary tumor tissues than in non-tumor tissues of same patients. Similar findigns were obtained with the RT-PCR test for mRNA levels. Decreased overall mRNA levels of TRPV5 and TRPV6 were associated with a worse overall survival rate (p=0.004 and p=0.003 respectively) and shorter recurrence-free survival (p<0.001 and p<0.001 respectively). The combining effect of TRPV5 and TRPV6 on survival was further investigated using multivariate analysis. The results showed that a combination of low expression of TRPV5 and TRPV6 could be an independent predictor of poor recurrence-free survival (p=0.002). Conclusions: Decreased expression of TRPV5/6 in tumor tissues was observed in NSCLC patients and was associated with shorter median survival time after surgical resection. Combined expression of TRPV5 and TRPV6 in tumor tissues demonstrated promising prognostic value in NSCLC patients.

Keywords: Non-small-cell lung cancer - TRPV5 - TRPV6 - prognostic marker

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Introduction

Lung is one of the four major cancer sites namely lung, breasts, colorectum and prostate. In the United States, lung cancer was expected to account for 26% and 28% of all cancer related deaths for female and male respectively in 2013. And in some Asia Countries, the recent incidence of lung cancer remained stable (Al-Hashimiand, 2014). It was estimated that there would be 228, 190 new cases and 159, 480 would die from lung cancer in the year of 2013 alone. The non-small cell lung cancer (NSCLC) accounts for 80-85% of all lung cancer cases, with the rest classified as small cell lung cancer (Herbst et al., 2008; Komaki et al., 2013). Lung cancer is always asymptomatic in its early stage and therefore in most cases it would not be diagnosed until it advances into terminal stage, bringing much more difficulty to treatment. These facts have made it important to look for reliable biomarkers for early diagnosis of lung cancer.

Previously known as CaT2 and CaT1 (Phelps et al., 2008), TRPV5 and TRPV6, the highly Ca^{2+}-selective channels, play a role in Ca^{2+} reabsorption in intestine and kidney (den Dekker et al., 2003; Hoenderop et al., 2003). Belonging to the transient receptor potential (TRP) superfamily along with six other subfamilies, namely, the TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin), TRPN (no mechanoreceptor potential C, NOMPC) and TRPP (polycystin), TRPV5 and TRPV6 are highly homologous members of the TRPV (vanilloid) subfamily (Vennekens et al., 2002; Ouadid-Ahidouch et al., 2013). The large and functionally versatile TRP superfamily consists of various cation channel proteins expressed in numerous cell types from yeast to mammals. (Lehen'kyi et al., 2012) These channels were first identified in *Drosophila melanogaster* (Minke, 1977; Montell et al., 1985).

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Numerous studies have shown that various cellular processes are regulated by calcium ion (Ca^{2+}) concentration, including cellular proliferation and apoptosis and that some Ca^{2+}-mediated signaling pathways are involved in tumorigenesis and tumor progression (Monteith et al., 2007). According to recent studies reported by Dhennin-Duthille et al. (2011), TRPV6 was mainly overexpressed in the invasive breast cancer cells, suggesting that TRPV6 may be useful as biomarker for breast cancer. In addition, the level of TRPV6 mRNA and/or protein is substantially elevated in human cancer cells from the thyroid, colon, prostate and ovary (Zhuang et al., 2002). Since Ca^{2+} flux is known as an outstanding mediator of both apoptotic and survival signaling mechanisms in tumor cells, it is reasonable to infer that TRPV6 protein might play functional roles in tumorigenesis, progression, or metastatic dissemination (ZhuangPeng et al., 2002).

The relationship between TRPV5/6, 1, 25 (OH)2D3, VDR and lung cancer is complex. Previous studies have shown that in the VDR knockout (VDR-/-) mouse, the serum level of 1, 25 (OH)2D3 is extremely high, presumably due to lack of catabolism (Henry, 2001; Nakagawa et al., 2005). The existence of 1, 25 (OH)2D3 in serum has been proven to inhibit the metastasis of lung cancer cells in a specific animal model (Nakagawa, et al. 2005). Besides, TRPV5 and TRPV6 share 74% amino acid identity and have several physiological functions in common. Although it is comforting that multicenter experiences have proved that adjuvant chemotherapy is standard for operable NSCLC, it has less clear the importance of this standard surgery for early stage lymph node positive NSCLC (Oven Ustaalioglu et al., 2013) and much less the molecular mechanism. And up to now, very few studies have focused on TRPV5 and TRPV6 in NSCLC; even fewer studies have investigated the roles of TRPV5 and TRPV6 in NSCLC. Therefore, in this study, we investigated the expression levels of TRPV5 and TRPV6 in NSCLC, and evaluated their prognostic significance on patients who has undergone surgical resection of NSCLC.

Materials and Methods

NSCLC and control specimens

NSCLC specimens were obtained from 145 NSCLC patients who had undergone surgical resection in Zhong Shan Hospital of Fudan University from 2008 to 2009. Patients' clinical characteristics were retrospectively collected (refer to Table 1). Pathologic staging was performed according to the TNM (Classification of Malignant Tumors) system (Ljungberg et al., 2007; UICC 2009). Fresh tumor samples were obtained during operation, and were preserved for mRNA test. The Institutional Review Board (IRB) committee of Zhong Shan hospital approved the acquisition and biomedical analysis of these samples. The informed consent forms were also signed by the patients and/or their family members.

Postoperative Treatment and Follow-up

Chemotherapies were conducted on the patients for 3 to 4 consecutive weeks following the operation, and all patients were followed up every 3 months for the first 2 years and every 6 months thereafter, till their death or the end of the follow up period. The follow-up was performed in clinic, by phone or by mail. With a median of 44 months (ranging from 2 to 60 months), the follow-up period ended on December 31, 2012. The treatment modality after relapse varied among individuals. Overall survival (OS) was defined as the interval between surgery and death, or between surgery and the last follow-up visit for surviving patients (Ju et al., 2009). The data were right censored at the last follow-up for surviving patients.

Tissue Immunohistochemical Analysis

Tissue immunohistochemical analysis was performed as described previously (Gao et al., 2007). All 145 samples from NSCLC patients were stained with hematoxylin and eosin. Photo results of each sample include duplicates of 1-mm-diameter cylinders from both tumor center and noncancerous margin, specified as intratumor and peritumor tissue with two punches respectively. The duplicates along with controls ensured reproducibility and homogenous staining. The tumor and non-tumor groups were thus formed, with each containing 290 slides. Sections of 4-um thickness were placed on slides coated with 3-aminopropyltriethoxysilane (Gao et al., 2007).

The primary antibodies were mouse monoclonal antibodies against TRPV5 (sc-23375, Santa Cruz Biotechnology, Santa Cruz, California) (1:200 dilution), and mouse monoclonal antibody against TRPV6 (sc-28763, Santa Cruz Biotechnology, Santa Cruz, California) (1:100 dilution). We underwent a two-step protocol (Novolink Polymer Detection System, Novocastra) to observe the immunohistochemistry of these paraffin sections according to the instructions of the manufacturer as described previously (Gao et al., 2007). First of all, paraffin sections were deparaffinized and hydrated conventionally. Then, after the retrieval of antigen using microwave heating, the activity of endogenous peroxidase was blocked by dipping slides into 0.3% H_2O_2, and the nonspecific antibody sites were blocked with Protein Block at room temperature overnight (RE7102; Novocastra). In the next step, slides were incubated with primary antibodies at room temperature for an hour, and blocked with Protein Block (RE7111; Novocastra) under the same condition after washing away the previous antibodies. At last, the secondary antibody (Novolink Polymer RE7112) was applied at 1:1000 concentration under the same condition. Through microscope, slide staining was controlled in diaminobenzidine solution and counterstained with hematoxylin in a timely manner. And in all assays, the negative control slides using no primary antibodies were included.

Immunohistochemical Scoring

Tissue microarray slides were concurrently evaluated by 2 independent pathologists, who were blinded to the patients' characteristics and none discrepancies were resolved by discussion. The intensity of TRPV5/TRPV6 immunoreactivity was graded according to staining of nuclear and cytoplasmic compartments of tumor cells. The

grading system was set as following: Grade 0, negative staining; Grade 1, 1-30% positive staining; Grade2, 30-60% positive staining; Grade 3, 60% positive staining. Only sample with grade≥2 was considered a positive immunohischemistry result, with grade=1 considered as week staining. Three microscope fields of one slide were observed and scored. The final score of a slide was the same as the score of the majority. Therefore, the number of fields with same score must at least exceed two in order to score this slide, or additional fields of the same slide would be selected for further observation until fields with the same score.

RT-PCR Analysis

Total RNA was isolated from normal lung tissues and NSCLC samples with an RN easy® Mini Kit and an RNase-Free DNase Set (Qiagen®). According to the manufacturer's instructions, approximately 0.5ug extracted RNAs were reversely transcribed with a First Strand cDNA Synthesis Kit for RT-PCR (Roche, Basel, Switzerland) with random primer. Real-time RT-PCR was performed with a Smart Cycler® System using SYBR® Green-I as the fluorogenic dye. 2μl cDNAs were added to a 25ul reaction system of ExTaq™ RT-PCR version with 0.2um of each pair of gene-specific primers and were subjected to 45 PCR cycles. Primer sequences were as followed: TRPV5 (GenBank® No.NM_019841.4, 185bp) (forward 5'-GGAGCTTGTGGTCTCCTCTG- 3' and reverse5'-GAAACTTAAGGGGGGCGGTAG-3'). TRPV6 (GenBank® No.NM_018646, 218bp) (forward 5'-CCCGAGGATTCCAGATGCTA-3') and reverse 5'-GATGATGGTAAGGAACAGCTCG- 3'). The thermal program lasted for 5 seconds at 95°C for denaturation, and 20 seconds at 60°C for annealing and elongation. Amplified PCR products were eletrophoresed on 2.5% agarose gel for verification.

Statistical analysis

Spss (17.0, Chicago, Illinois, USA) statistical package was employed for data analyses. Association between clinicopathological characteristics and protein expression level was evaluated using chi-squared test. Kaplan-Meier method was used to evaluate recurrence-free survival (RSF) and OS, for which only patients with strong expression of both TRPV5 and TRPV6 (grade 2 and above for immunohistochemical scoring) are categorized as TRPV5/6 positive with the rest categorized as TRPV5/6 negative. In this study, α level of 0.05 was chosen for the statistical analysis.

Results

The Expression of TRPV 5 and TRPV 6 in cancer tissues and corresponding normal tissues

In RT-PCR analysis, TRPV5 mRNA was less expressed in tumor tissue than in normal tissue (0.00008935±0.00001347 versus 0.00026770±0.00010989, p=0.013). Similar findings were observed in TRPV6 mRNA level (0.0006899±0.0000846 versus 0.001374±0.0001783, p=0.022).

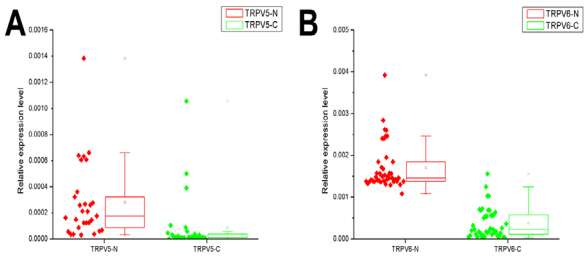

Figure 1. RT-PCR Analysis of TRPV5 and TRPV6 Expression Level in Intra-Tumor (Tumor Center) and Paritumor (Noncancerous margin) Tissues

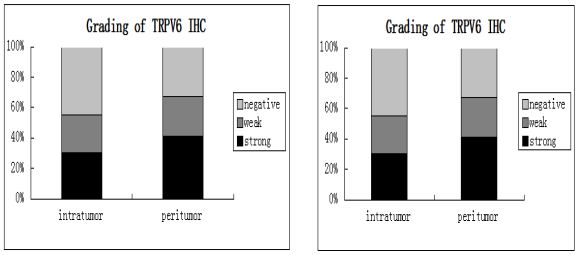

Figure 2. Grading of TRPV5 and TRPV6 Staining in Intratumor and Paritumor Tissues

Immunohistochemical analysis of NSCLC tumor samples

In tissue immunohistochemical analysis, (representative images of different grades of staining are shown in Figure 3), TRPV5 and TRPV6 staining were found to be remarkably lower in cancer tissues than in control peritumor tissues. The distribution of different grades of TRPV5 and TRPV6 staining is illustrated in Figure 2. NSCLCs were featured with significantly lower average grading of TRPV5 and TRPV6 compared with peritumor tissues from the same parents in pair (p=0.00021 for TRPV5 and p=0.00062 for TRPV6).

Prognostic factors

Within the 5 years of follow-up, 71 patients died, including 6 patients who died of other causes without record of tumor recurrence. During the follow-up period with a median duration of 44 months (44.3±7.4), 39 patients had tumor metastases or recurrence, and the 5-year overall survival rate was 51.03% for the entire study population.

In univariate analysis, age, sex, histological type, and tumor differentiation were unfavorable predictors for OS and RFS, while TNM stage showed prognostic significance for both OS and RFS (p=0.031, 0.044, respectively). Lower TRPV5 or TRPV6 expression had no oncological value as analyzed by IHC, but decreased mRNA level of TRPV5 and TRPV6 was associated with a worse overall survival rate (p=0.004, p=0.013, respectively), and was correlated with a shorter recurrence-free survival rate (p<0.001, p<0.001, respectively; Table 1).

The combined effect of TRPV5 and TRPV6 on the survival of NSCLC patients was also evaluated in this study. According to the IHC data of TRPV5 and TRPV6, patients were classified into two groups: those who were either TRPV5 positive or TRPV6 positive (immunohistochemical score 2+) were marked as TRPV+

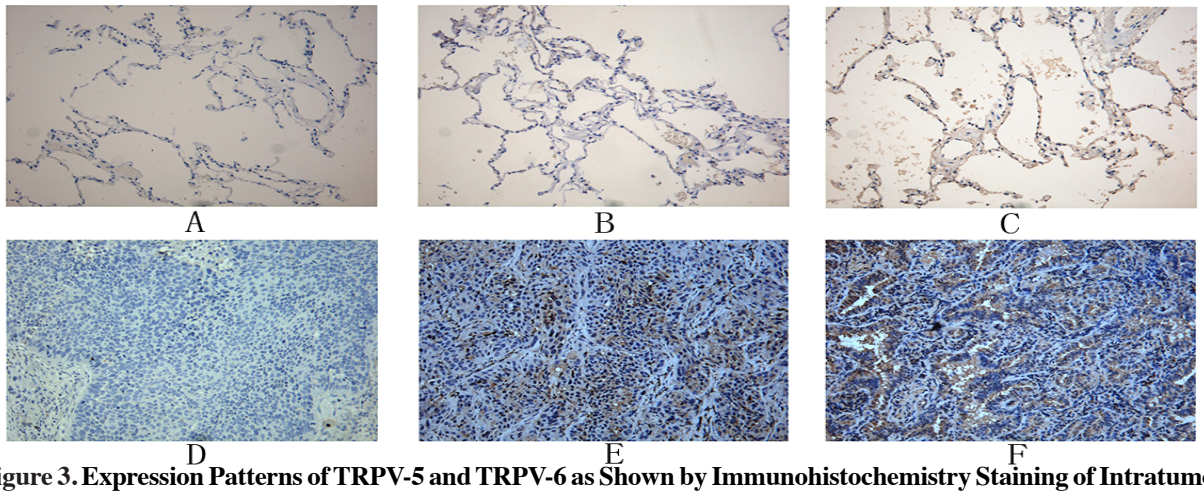

Figure 3. Expression Patterns of TRPV-5 and TRPV-6 as Shown by Immunohistochemistry Staining of Intratumor and Peritumor Tissues. Representative examples of **A)** strong staining, **B)** weak staining and **C)** negative staining

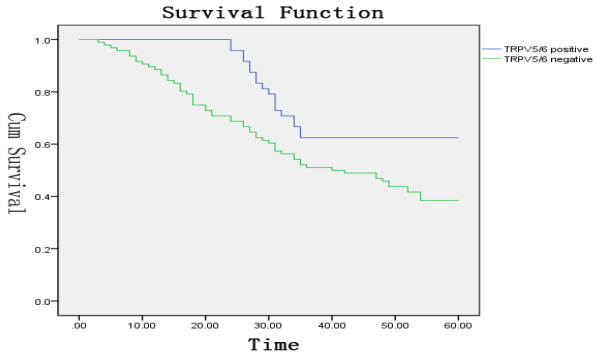

Figure 4. Survival Curve of TRPV5/6 Negative and Positive Patients Groups

Table 1. Patient Characteristics

Clinical Features			
Age (years)	61.2		
Sex (Male/Female)	87	58	
Histology (Adeno/Squamous)	80	65	
Differentiation (High/Medium/Low)	31	47	67
Smoker (none/ever)	52	88	
TRPV5 (positive/negative)	26	119	
TRPV6 (positive/negative)	44	101	

Table 2. Correlation between TRPV5 and TRPV6 by IHC

TRPV	TRPV5+	TRPV5-
TRPV6+	21	23
TRPV6-	5	96

(49/145, 33.79%) with the rest marked as TRPV- (96/145, 66.21%). TRPV5/6 were not associated with RFS (data not shown), but there was statistically significant correlation between their level and the OS (p=0.018) (Figure.4).

On multivariate analysis, clinicopathologic characteristics were adopted as covariates when multivariate Cox proportional hazards analysis was performed. We found that TNM stage and TRPV+/- were independent prognostic factors for RFS (p=0.003, p=0.004, respectively) when compared with TRPV5/6 using IHC or RT-PCR analysis. (Table 2).

Correlation with Clinicopathologic Variables

Decreased expression of TRPV5 and TRPV6 was not significantly related with any clinicopathologic variables, age, gender, TNM stage, or histological type.

Discussion

The clinical significance of TRPV5 and TRPV6 has been implicated in certain types of human cancers (Vriens et al., 2004). In this study, we found that both mRNA and protein level of TRPV5/6 were significantly decreased in NSCLC compared with normal tissue from the same patients (peritumor), and such decrease was correlated with poor OS and RFS in patients who had undergone surgical resection of NSCLCs. Co-existence of both markers indicated positive survival possibility in patients with staged NSCLC, and was an independent prognostic indicator for both OS and RFS in multivariate analysis.

In recent years, TRPV5 and TRPV6 have boosted the research addressing epithelial Ca^{2+} transport (Vanoevelen et al., 2011). In addition to their role in maintaining the calcium homeostasis (de Groot et al., 2011), some reports have also suggested TRPV5 and TRPV6 as important biomarkers in tumor development and progression (Heise et al., 2010). This has been proved in prostate, breast, thyroid, colon, pancreatic and ovary cancers. However, few evidences exist in terms of TRPV5 or TRPV6 as potential biomarker in the carcinogenesis of NSCLC. In this report, decreased TRPV5 and TRPV6 protein level was observed from lung cancer samples using IHC, and at meantime, decreased TRPV5 and TRPV6 mRNA level was observed from RT-PCR assay. These findings might be explained by the physiological disorder caused by tumor progression. Similar findings were observed in the report of Wu etc. on renal cancer [23].

In the publication by Schwarz etc., the alteration in calcium homeostasis would increase proliferation and induce differentiation (Schwarz et al., 2006). Since TRPV5 and TRPV6 are highly selective Ca^{2+} channels, the calcium homeostasis might be well broken during tumor progression. The increase in [Ca^{2}] Ion channel

permeability and mitochondrial [Ca^2] could activate mitochondrial membrane permeabilization and lead to apoptosis and necrosis. In contrast, the decrease in Ca^2 contents of the endoplasmic reticulum lumen is associated with resistance to apoptosis (Ahmad et al., 2009). In this study, changes in TRPV and its association with cancer differentiation were noticed, as observed in other reports (Dhennin-Duthille et al., 2011). The result from RT-PCR test was consistent with IHC data, indicating that certain signal transduction pathways related with TRPV5/6 might have been affected. Further research needs to be performed in order to answer the following question: which pathway (s) related with TRPV5/6 is (are) affected by tumor development, and how are they affected to exert their alternated role on cell development?

In other reports, the prognostic values of TRPV5 and TRPV6 were also studied (Hartel et al., 2006), and the results remained controversial. In this study, the level of TRPV5 and TRPV6 showed a tendency of being associated with patients' survival. However, the prolonged survival period was not significantly associated with TRPV 5 or TRPV6 alone. Only those patients who were both TRPV5 and TRPV6 negative in IHC experienced shorter duration of survival. Given the relatively small sample size of the study, the independent prognostic value of TRPV5 or TRPV6 in NSCLCs remained unclear. TRPV5/6 could be a promising tool for cancer diagnosis and even cancer therapy in the future.

To our knowledge, this is the first study of TRPV5/6 as specific prognostic indicators in NSCLC patients, and we have demonstrated the prognostic significance of TRPV5/6 in NSCLC. The study may provide valuable prognostic information and help to reveal novel treatment targets for future NSCLC case management. Since the study was limited in its retrospective design and small case volumes, further research is needed to evaluate the significance of these findings.

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