Preserved SCN4B expression is an independent indicator of favorable recurrence-free survival in classical papillary thyroid cancer

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Abstract

Voltage-gated sodium channel β subunits (encoded by SCN1B to SCN4B genes) have been demonstrated as important multifunctional signaling molecules modulating cellular processes such as cell adhesion and cell migration. In this study, we aimed to explore the expression profiles of SCN4B in papillary thyroid cancer (PTC) and its prognostic value in terms of recurrence-free survival (RFS) in classical PTC. In addition, we also examined the potential effect of DNA methylation on its expression. A retrospective study was performed by using data from available large databases, including the Gene Expression Omnibus (GEO) datasets and the Cancer Genome Atlas (TCGA)-Thyroid Cancer (THCA). Results showed that SCN4B is downregulated at both RNA and protein level in PTC compared with normal thyroid tissues. Preserved SCN4B expression was an independent indicator of favorable RFS in patients with classical PTC, no matter as categorical variables (HR: 0.243, 95%CI: 0.107–0.551, \( p = 0.001 \)) or as a continuous variable (HR: 0.684, 95%CI: 0.520–0.899, \( p = 0.007 \)). The methylation status of one CpG site (Chr11: 118,022,316–318) in SCN4B DNA had a moderately negative correlation with SCN4B expression in all PTC cases (Pearson's \( r = -0.48 \)) and in classical PTC cases (Pearson's \( r = -0.41 \)). In comparison, SCN4B DNA copy number alterations (CNAs) were not frequent and might not influence its mRNA expression. In addition, no somatic mutation was found in SCN4B DNA. Based on these findings, we infer that preserved SCN4B expression might independently predict favorable RFS in classical PTC. Its expression might be suppressed by DNA hypermethylation, but is less likely to be influenced by DNA CNAs/mutations.

Introduction

Voltage-gated sodium channels are integral membrane proteins that constitute one large pore-forming principal α subunit and one or two smaller transmembrane β subunits as auxiliary (encoded by SCN1B to SCN4B genes) [1, 2]. Although the β subunits were firstly identified as...
auxiliary subunits modulating the gating, kinetics, and localization of the ion channel pore, there are emerging studies showed that they are also important multifunctional signaling molecules regulating cell adhesion, cell migration, differentiation, endosome acidification, phagocytosis and podosome formation, with or without the presence of pore-forming α subunit [2, 3]. Some recent studies found that the sodium channel β subunits are dysregulated in oncogenic processes. SCN1B expression is decreased in highly metastatic breast cancer cells lines [4], but is increased in highly metastatic prostate cancer cell lines [5]. In breast cancer cells, decreased SCN4B protein expression correlates with high-grade primary and metastatic breast tumors and is also associated with enhanced breast cancer cell migration, invasiveness and metastatic spreading [6].

Papillary Thyroid Cancer (PTC) is the dominant form of thyroid cancer, which is usually indolent in progression [7, 8]. The standard treatment of PTC is total thyroidectomy or hemithyroidectomy with following radioiodine ablation and thyrotropin suppression in appropriately selected cases. Patients after these treatments generally have a favorable prognosis, with a 10-year survival rate over 95% [8]. However, disease recurrence and/or distant metastasis were observed in about 5–20% of the patients, which lead to aggressive and lethal outcomes [7, 9]. PTC consists of several histological subtypes, such as classical/usual PTC, follicular PTC, and tall-cell PTC, among which the classical PTC is the most prevalent subtype. These subtypes have different biological behaviors and may have different clinical implications [7, 9]. Therefore, it is meaningful to explore the biomarker of recurrence in different histological subtypes.

In this study, by using data from the Cancer Genome Atlas (TCGA)-Thyroid Cancer (THCA), we performed a retrospective study to explore the expression profiles of SCN4B in PTC and its prognostic value in terms of recurrence-free survival (RFS) in classical PTC. In addition, we also examined the potential effect of DNA methylation on its expression.

**Materials and methods**

**Secondary analysis of microarray data in GEO datasets**

In the Gene Expression Omnibus (GEO) datasets, one previous Affymetrix Human Genome U133 Plus 2.0 Array (GSE3678) analyzed the gene expression profiles of 7 PTC samples compared to 7 paired normal samples. The raw SOFT data file of this array was downloaded and reanalyzed to identify the expression profile of sodium channel subunits.

**SCN4B Immunohistochemistry (IHC) staining**

SCN4B IHC staining in normal thyroid and PTC tissues was examined in the Human Protein Atlas (http://www.proteinatlas.org/) [10, 11], which is an online tool for genome-wide analysis of the human proteins.

**Retrospective analysis using data from TCGA-THCA**

The association between SCN4B expression and the clinicopathological parameters and RFS in PTC patients was studied by performing a retrospective analysis using the level 3 data of TCGA-THCA. Data mining was performed as introduced by one previous study [12]. According to the description by TCGA, the pathological assessment of the biospecimens was performed a board-certified pathologist to ensure the accuracy [13]. Tumors were classified as the follicular variant if it were 99% follicular patterned, and as the tall cell variant if it had 50% or greater tall cell features [13]. In brief, the original data, including sample type, age at initial pathologic diagnosis, histological types, gender, pathological stage, lymph nodal invasion, residual tumors, the history of radiation therapy, recurrence status and RFS in days were
obtained by using the UCSC Xena Browser (https://xenabrowser.net/). In this patient cohort, the tumor tissue from 505 PTC cases (358 classical/usual cases, 102 follicular cases, 36 tall cell cases and 4 cases not specified) and 59 normal thyroid tissues were subjected to RNA-seq (by IlluminaHiSeq). 358 out of the 505 PTC cases belong to classical/usual histological subtype. 348 out of the 358 classical PTC cases had RFS data recorded and were subjected to survival analysis. The flowchart showing the inclusion of patients was given in S1 Fig. Kaplan-Meier curves of RFS were generated by using GraphPad Prism 6.0 (GraphPad Inc.).

The SCN4B DNA methylation data (Illumina 450k infinium methylation beadchip), Gene-level thresholded GISTIC2-processed DNA copy number alterations (CNAs) data, as well as DNA mutation data (SNPs and small insertions and deletions (INDELs)) were downloaded to investigate the potential mechanisms of SCN4B dysregulation in PTC.

Statistical analysis

Data were reported as means ± standard deviations (SDs). Statistical analysis was performed by using Prism 6.0 or SPSS 19.0 software package (SPSS Inc.). Welch’s unequal variances t-test was applied to compare the difference in SCN4B expression between groups with different clinicopathological parameters. Receiver operating characteristic (ROC) analysis for recurrence detection was applied to identify the best cut-off (Youden index) for SCN4B expression in survival analysis.

Chi-square tests were performed to compare the association between SCN4B expression and the clinicopathological parameters. Kaplan-Meier curves of RFS was generated using GraphPad Prism 6.0. Patients were grouped by setting the Youden index as the cut-off. Log-rank test was performed to assess the significance of the difference between the survival curves. Univariate and multivariate Cox regression models were used to evaluate the independent prognostic value of SCN4B expression (as either categorical variables or a continuous variable) in terms of RFS. p<0.05 was considered statistically significant.

Results

SCN4B was downregulated in PTC compared with normal thyroid tissues

Using the raw data of one previous array (GSE3678), we examined the expression profiles of sodium channel subunits between normal thyroid tissues and PTC tissues (Fig 1A). The heatmap of the array results showed that SCN4B was one of the most downregulated sodium channel subunits in PTC compared with normal thyroid tissues (Fig 1A, red dotted frame). To verify this dysregulation, we also examined its expression by using RNA-seq data in TCGA-THCA. In this cohort, 505 primary PTC tissues and 59 normal thyroid tissues were subjected to RNA-seq (Fig 1B). Heatmap and the following comparison showed that SCN4B was significantly reduced in PTC tissues (p<0.001, Fig 1B and 1C). Then, using human tissue IHC staining results in the HPA, we also examined SCN4B protein expression in normal thyroid and PTC tissues. The staining results indicated that normal thyroid usually had moderate SCN4B expression (Fig 1D). In comparison, among 4 cases of PTC tissues examined, only two cases had low SCN4B staining, while the rest two cases had negative SCN4B expression (Fig 1E). These findings suggest SCN4B is downregulated at both RNA and protein level in PTC compared with normal thyroid tissues.

Decreased SCN4B expression was associated with recurrence in classical PTC

One previous study reported that SCN4B is a metastasis-suppressor gene in breast cancer [6]. In this study, we also examined the association between SCN4B expression and metastasis and
lymph nodal invasion in PTC. Results showed that there was no significant difference in SCN4B expression between the cases with or without metastasis (Fig 2A). However, the lymph nodal positive cases had substantially decreased SCN4B expression compared to their counterparts (Fig 2B). Using the survival data in TCGA-THCA, we also examined the recurrence status of different histological PTC cases. Heatmap showed that the classical/usual PTC subtype had the largest proportion of recurrence (Fig 2C, red dotted frame). The cases with recurrence also had significantly decreased SCN4B expression ($p = 0.012$, Fig 2D).

Preserved SCN4B expression is an independent indicator of favorable RFS in classical PTC

By generating Kaplan-Meier curves of RFS in classical PTC patients, we found that high SCN4B expression was associated with significantly better RFS ($p < 0.001$, Fig 3). The clinicopathological parameters in the high and low SCN4B expression groups were summarized in Table 1. Chi-square analysis showed that the high SCN4B expression was associated with a significantly lower ratio of nodal invasion (115/227, 50.7% vs. 61/96, 63.5%, $p = 0.034$) and recurrence (9/243, 3.7% vs. 16/105, 15.2%, $p < 0.001$) compared with the low SCN4B expression group (Table 1). In univariate COX regression analysis, low pathological stage, no residual tumors and high SCN4B expression (as categorical variables)/increased SCN4B expression (as a continuous variable) were associated with favorable RFS (Tables 2 and 3). In the following multivariate analysis, preserved SCN4B expression was an independent indicator of favorable
RFS, no matter as categorical variables (HR: 0.243, 95%CI: 0.107–0.551, \( p = 0.001 \)) (Table 2) or as a continuous variable (HR: 0.684, 95%CI: 0.520–0.899, \( p = 0.007 \)) (Table 3).
DNA hypomethylation might be a mechanism of decreased $SCN4B$ expression in PTC

In Illumina 450k infinium methylation beadchip, the methylation status of 27 CpG sites in $SCN4B$ DNA was measured. By comparing $SCN4B$ expression and its DNA methylation, we found that the methylation status of one CpG site (Chr11: 118,022,316–318) was negatively correlated with $SCN4B$ expression (Fig 4A). Regression analysis confirmed a moderately negative correlation in all PTC cases (Pearson’s $r = -0.48$) (Fig 4B). In classical PTC cases, this negative correlation was also confirmed (Pearson’s $r = -0.41$) (Fig 4C). Then, we also examined the association between $SCN4B$ RNA expression and its DNA CNAs/mutations. Results showed that among 505 primary PTC cases, 497 cases had CNAs measured, while only 14 cases had CNAs (9 heterozygous loss (-1) and 5 low-level copy gain (+1)) (S2A and S2B Fig). These alterations did not influence $SCN4B$ expression (S2A and S2B Fig). Besides, no somatic mutation was found in $SCN4B$ DNA (S2A Fig).

### Table 1. The association between $SCN4B$ expression and the clinicopathological parameters in patients with classical PTC.

| Parameters       | $SCN4B$ expression | $\chi^2$ | $p$ |
|------------------|--------------------|----------|-----|
|                  | High (N = 243)     | Low (N = 105) |
| Age (Mean ± SD)  | 46.61±15.84        | 45.71±16.95  | N.A. | 0.64 |
| Gender           |                     |           |     |
|                  | Female              | 175       | 77  | 0.06 | 0.80 |
|                  | Male                | 68        | 28  |      |      |
| Pathological Stage| III/IV             | 75        | 36  | 0.40 | 0.53 |
|                  | I/II                | 168       | 69  |      |      |
| Nodal invasion   | No                  | 112       | 35  | 4.51 | 0.034|
|                  | Yes                 | 115       | 61  |      |      |
|                  | NX/No data          | 16        | 9   |      |      |
| Residual tumors  | R0                  | 183       | 81  | 0.004| 0.95 |
|                  | R1/R2               | 30        | 13  |      |      |
|                  | RX/no data          | 30        | 11  |      |      |
| Radiation therapy| No                  | 90        | 40  | 0.025| 0.87 |
|                  | Yes                 | 145       | 62  |      |      |
|                  | No data             | 8         | 3   |      |      |
| Recurrence status| No                  | 234       | 89  | 14.63| <0.001|
|                  | Yes                 | 9         | 16  |      |      |

NX: Regional lymph nodes cannot be assessed; R0: No residual tumor; R1: Microscopic residual tumor; R2: Macroscopic residual tumor; RX: The presence of residual tumor cannot be assessed.

DNA hypomethylation might be a mechanism of decreased $SCN4B$ expression in PTC

Table 2. Univariate and multivariate analysis of RFS in patients with classical PTC ($SCN4B$ expression as categorical variables).

| Parameters       | Univariate analysis | Multivariate analysis |
|------------------|---------------------|-----------------------|
|                  | $\chi^2$ | $p$ | HR | 95%CI (lower/upper) | $\chi^2$ | $p$ | HR | 95%CI (lower/upper) |
| Age (Continuous)                   | 0.276 | 0.103 | 1.013 | 0.990 | 1.037 |
| Gender Female vs. Male             | 0.501 | 0.749 | 0.323 | 1.737 |
| Clinical stage III/IV vs. I/II     | 0.035 | 2.329 | 1.061 | 5.113 | 0.139 | 1.872 | 0.816 | 4.296 |
| Nodal invasion No vs. Yes          | 0.843 | 0.920 | 0.403 | 2.099 |
| Residual tumors No vs. Yes         | 0.014 | 0.332 | 0.137 | 0.800 | 0.052 | 0.398 | 0.157 | 1.009 |
| $SCN4B$ expression (High vs. Low)  | 0.001 | 0.240 | 0.106 | 0.544 | 0.001 | 0.243 | 0.107 | 0.551 |

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Discussion

There are emerging studies showed that dysregulated sodium channel β subunits are implicated in multiple types of cancer. In mouse model bearing implanted prostate tumor, SCN1B expression is associated with enhanced growth rate and size, as well as decreases in survival rates [5]. In breast cancer, enforced SCN1B expression could promote pathological growth and cellular dissemination, including metastasis to both lung and liver [14]. In cervical cancer tissues, SCN3B mRNA level is increased, whereas SCN1B, SCN2B and SCN4B mRNA levels are decreased [15]. SCN3B is induced in mouse embryonic fibroblasts by DNA damage in a p53-dependent manner and mediates a p53-dependent apoptotic pathway [16]. In breast cancer cells, reduced SCN4B expression is associated with increased RhoA activity, enhanced cell migration and invasiveness, primary tumor growth and metastatic spreading, via promoting

Table 3. Univariate and multivariate analysis of RFS in patients with classical PTC (SCN4B expression as a continuous variable).

| Parameters             | Univariate analysis | Multivariate analysis |
|------------------------|---------------------|-----------------------|
|                        | p       | HR   | 95% CI (lower/upper) | p       | HR   | 95% CI (lower/upper) |
| Clinical stage III/IV vs. I/II | 0.035  | 2.329 | 1.061/5.113          | 0.115  | 1.943 | 0.850/4.444          |
| Residual tumors No vs. Yes | 0.014  | 0.332 | 0.137/0.800          | 0.064  | 0.417 | 0.165/1.051          |
| SCN4B expression (Continuous) | 0.005  | 0.681 | 0.522/0.889          | 0.007  | 0.684 | 0.520/0.899          |

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Fig 4. DNA hypomethylation might be a mechanism of decreased SCN4B expression in PTC. A. Heatmap showing the correlation between SCN4B expression and DNA methylation (Methylation 450k) in different subtypes of PTC. B-C. Regression analysis of the correlation between SCN4B expression and DNA methylation in all PTCs (B) and classical subtypes (C).

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the acquisition of an amoeboid-mesenchymal hybrid phenotype [6]. These results suggest that the expression and functional role of sodium channel β subunits might be tissue specific.

In this study, by using available large databases, we examined the expression profiles of sodium channel subunits in PTC and found that SCN4B was significantly downregulated at both mRNA and protein levels in PTC compared with normal thyroid tissues. In addition, we also observed that the PTC cases with lymph nodal invasion had significantly lower SCN4B expression. In classical PTC subtype, we confirmed the association between decreased SCN4B expression and the risk of recurrence. PTC patients usually have long-term overall survival and the primary goal of surgery is to minimize the risk of local recurrence and distant metastasis [17]. Therefore, we decided to further investigate the potential prognostic value of SCN4B expression in terms of RFS in the classical subtype. By generating Kaplan-Meier curves of RFS, we found that the high SCN4B expression group had significantly better RFS. The following univariate and multivariate analysis confirmed that preserved SCN4B expression was an independent indicator of favorable RFS in patients with classical PTC, no matter as categorical variables (HR: 0.243, 95%CI: 0.107−0.551, \( p = 0.001 \)) or as a continuous variable (HR: 0.684, 95% CI: 0.520−0.899, \( p = 0.007 \)). These findings suggest that SCN4B might be a promising prognostic biomarker in classical PTC.

Genetic mutation and epigenetic alteration (such as DNA hypomethylation) are important mechanisms leading to suppressed transcription of some important tumor suppressors in cancers, including PTC [18, 19]. For example, RASAL1 is a major tumor suppressor gene in thyroid cancer, which is frequently inactivated by hypermethylation and mutations [20]. ZIC1 is also a tumor suppressor gene in thyroid cancer by blocking the activities of the PI3K/Akt and MAPK signaling pathways and the transcription of transcription factor FOXO3a [21]. However, it is frequently inactivated by promoter hypermethylation [21]. CDH1 and SCL5A8 promoter methylation are also associated with the carcinogenesis of thyroid tumor [22]. In this study, by examining the methylation status of 27 CpG sites in SCN4B DNA, we found that the methylation status of one CpG site (Chr11: 118,022,316–318) had a moderately negative correlation with SCN4B expression in all PTC cases (Pearson’s \( r = -0.48 \)) and in classical PTC cases (Pearson’s \( r = -0.41 \)). In comparison, SCN4B DNA CNAs were not frequent and might not influence its mRNA expression. In addition, no somatic mutation was found in SCN4B DNA. These findings suggest that DNA hypermethylation might be an important mechanism of suppressed SCN4B expression in PTC.

Conclusion

In summary, findings in the study showed that SCN4B is downregulated in PTC compared with normal thyroid tissues. Preserved SCN4B expression might independently predict favorable RFS in classical PTC. Its expression might be suppressed by DNA hypermethylation, but is less likely to be influenced by DNA CNAs/mutations.

Supporting information

S1 Fig. The flowchart showing the inclusion of patients. (JPG)

S2 Fig. SCN4B DNA CNAs and mutations. A. Heatmap showing the correlation between SCN4B expression and its DNA CNAs and mutations in different subtypes of PTC. B. Plots chart showing SCN4B expression in heterozygous loss (-1), copy-neutral (0) and low-level copy gain (+1) groups. (JPG)
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