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ORIGINAL PAPER

Genetic copy number alterations and IL-13 expression differences in papillary thyroid cancers and benign nodules

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Abstract Thyroid nodules were the extremely common endocrine tumors, in which papillary thyroid carcinomas (PTCs) were the most prevalent endocrine malignancy, representing 80–90% of all thyroid malignancies. It was still a dilemma to discriminate PTCs and benign thyroid nodules. With a new molecular genetics technology of Multiplex ligation-dependent probe amplification (MLPA), we investigated 13 PTC and 14 benign nodule tissue samples. The results showed that PTCs had more genetic copy number alteration than benign nodules ($P < 0.001$). Receiver operating characteristic (ROC) curve analysis suggested that genomic aberrations would provide a moderate accuracy method to discriminate PTCs and benign nodules. The gain of interleukin 13 (*IL-13*) gene obviously identified the great difference between PTCs and benign nodules. Immunohistochemistry also confirmed significantly higher IL-13 expression in the PTCs ($P < 0.001$). The current study showed that MLPA should be an effective method to diagnose PTCs and benign thyroid nodules, and also provided a clue to another relationship between IL-13 and PTCs.

Keywords Papillary thyroid carcinomas (PTCs) · IL-13 · Multiplex ligation-dependent probe amplification (MLPA)

Introduction

Thyroid nodules were the extremely common endocrine and 5–10% of the population would develop a clinically significant thyroid nodule during their life [1]. However, only about 5% clinically identified nodules were malignant [2]. Papillary thyroid carcinomas (PTCs) were the most prevalent endocrine malignancy, representing 80–90% of all thyroid malignancies [3]. Fine needle aspiration biopsy (FNA) was the best test to evaluate a patient with a benign or malignant thyroid nodule. However, 10–25% of FNAs were not definitively diagnosed as benign or malignant [4].

During the past decades, significant progress has been made in the identification of genetic alterations associated with PTCs. The frequent genetic alterations in PTCs were ret proto-oncogene (*RET/PTC*) rearrangements and v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*), which are more common in radiation-induced childhood PTCs [5, 6] and adult sporadic PTCs, respectively, [7, 8]. Gene copy number alterations were also associated with particular types of human tumor. With the assay of comparative genomic hybridization (CGH), loss of chromosomes 1p, 3p, 3q, 11p, 17p, 22q and gain of chromosomes 17q, 11q13 were identified in the different PTCs studies

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[9–13]. However, CGH analysis was difficult to identify the specific gene involved in the pathogenesis. Meanwhile, it was difficult for this method to be used as a routine practice because it was laborious and needed relatively large amount of DNA [14, 15]. Therefore, we tried to develop a new technology, multiplex ligation-dependent probe amplification (MLPA), to detect genetic alterations in PTCs and benign nodules. MLPA was a robust assay for detecting copy number changes in chromosomes. This method relied on sequence-specific probe hybridization to genomic DNA followed by multiplex-PCR amplication of the hybridized probe, and semi-quantitative analysis of the resulting PCR products. Comparing with CHG, MLPA can detect genetic copy number changes not only in chromosomes but also in the genes associated with tumors; meanwhile, it was easy to perform and requires only 50 ng of DNA [14, 15].

Materials and methods

Patients and samples

The thyroid tumor tissues used in MLPA were obtained from 13 papillary thyroid cancer patients (10 women and 3 men; mean age 45.90±13.48 years; range 27–72 years) and 14 thyroid benign nodule patients (all women; mean age 51.43±11.28 years; range 37–69 years). In our study, the female-to-male ratio of thyroid nodules is higher than other Chinese studies' reported ratio of 4:1 [16, 17]. All the patients have accepted thyroidectomy in 2000–2006 at general surgery department in Ruijin Hospital. PTC or benign nodule tissue blocks were stored in liquid nitrogen immediately after being dissected by pathologists. The final diagnosis was obtained from the pathology report. The sections for immunohistochemistry were obtained from pathology department of Ruijin Hospital. This study was approved by the Ethics Committee of the Ruijin Hospital, Shanghai JiaoTong University School of Medicine.

MLPA reaction

Genomic DNA was extracted from fresh-frozen tumor specimens using a commercially available kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. We performed MLPA analysis using SALSA P005 and P006 Chromosomal Aberration MLPA Kits (MRC-Holland, Amsterdam, The Netherlands; details available at http://www.mlpa.com) following the manufacturer's protocol. The P005 and P006 kits include a total 76 genes, which were reported to be involved in cancer and could span all 23 chromosomes including the X and the Y. Briefly, 5 μl of DNA samples were heated at 98°C for 5 min; after the addition of the probe mix, samples were heated for 1 min at 95°C and then incubated at 60°C for 16 h. Ligation of the annealed oligonucleotide probes was performed for 15 min at 54°C with Ligase-65 enzyme. Multiplex PCR amplification was carried out using Cy5-labeled primers, dNTPs, and SALSA polymerase. PCR was performed for 35 cycles of 30 s at 95°C, 30 s at 60°C, and 1 min at 72°C. All the reactions were carried out in PTC-225 DNA Engine Tetrad (MJ Research Inc., San Francisco, CA, USA). PCR products were analyzed using Beckman Coulter CEQ 8800 sequencer (Beckman Coulter, Fullerton, CA, USA).

MLPA data analysis

Data analysis was performed with fragment analysis module. All the samples showed low peaks of ligation-independent control peaks, indicating that sufficient amounts of DNA were obtained for reliable analysis. Normal control DNA of males and females was used in the same reaction. The Gene Scan data of sizes and peak height of multiplex PCR products were exported to an Excel file. All the expected MLPA products were normalized by dividing each peak height by the combined peak height of all peaks in that lane (relative peak height). The relative copy number for each probe was expressed as a ratio of the relative peak height for each locus of the sample to that of the normal sex-matched control. The reference median peak heights were obtained from normal tissue samples, each of which was analyzed at least three times independently. Male and female samples were compared with male and female control samples, respectively, for quantitation of X- and Y-linked probes. The ratio <0.7 or >1.3 was considered as a loss and a gain, respectively. The numbers of loss and gain were analyzed to reveal the genetic copy differences in PTCs and benign thyroid nodules.

Immunohistochemistry

Sections of 4–5 μm were cut from paraffin blocks and heated at 60°C over night. Slides were deparaffinized and re-hydrated. Then the sections were immersed in sodium citrate buffer adjusted to pH 6.0 and heated in a microwave oven for 5 min at 100°C. They were then treated with 0.3% H_2O_2 in methanol for 30 min at room temperature to inhibit peroxidases activity. To reduce non-specific background staining, slides were incubated with 2% goat serum for 10 min at room temperature. Slides were then incubated with goat monoclonal antibody against human interleukin 13 (IL-13) (Santa Cruz Bio. Inc.) diluted to 1:150 in a moist chamber overnight at 4°C. The primary antibody binding was demonstrated with goat ABC staining system (Santa Cruz Bio. Inc.). Peroxidase activity was detected with diaminobenzidine as the substrate. Finally, sections

were weakly counterstained with Harris's hematoxylin and mounted. Negative controls were incubated without primary antibody. The results were evaluated quantitatively and divided into four groups (+++, >60%; ++, 30–60%; +, <30%; −, negative). The data were entered into a ZEISS Axioplan 2 microcomputer. All samples were treated and examined under identical conditions.

Statistical analysis

The total numbers of loss and gain in PTCs and benign thyroid nodules were analyzed using SPSS 11.0 software. Chi-Square test was used to assess the difference in the number of genetic alterations between the two experiments. Receiver operating characteristic (ROC) curve analysis was used to assess the validity of the MLPA analysis in a diagnostic setting. Statistical analysis about immunohistochemistry results was also carried out using SPSS11.0 software. The Mann–Whitney *U*-test was used to estimate significances of the percentage of IL-13 immunoreactivity in PTC and benign nodule samples. $P < 0.05$ was taken as level of significance.

Result

MLPA

The genomic aberrations were detected in all samples, including both PTCs and benign nodules. PTCs showed multiple copy number losses and gains, the total number of aberrations ranging from 6 to 27 and the average number being 17.08±6.12 (Fig. 1). By contrast, benign nodules showed few genetic changes, the number of aberrations ranging from 3 to 24 and average number being 9.07±3.85. Totally, 23% (222/968) PTC samples and 12% (127/1036) benign nodules had the significant difference between the two groups ($P < 0.001$).

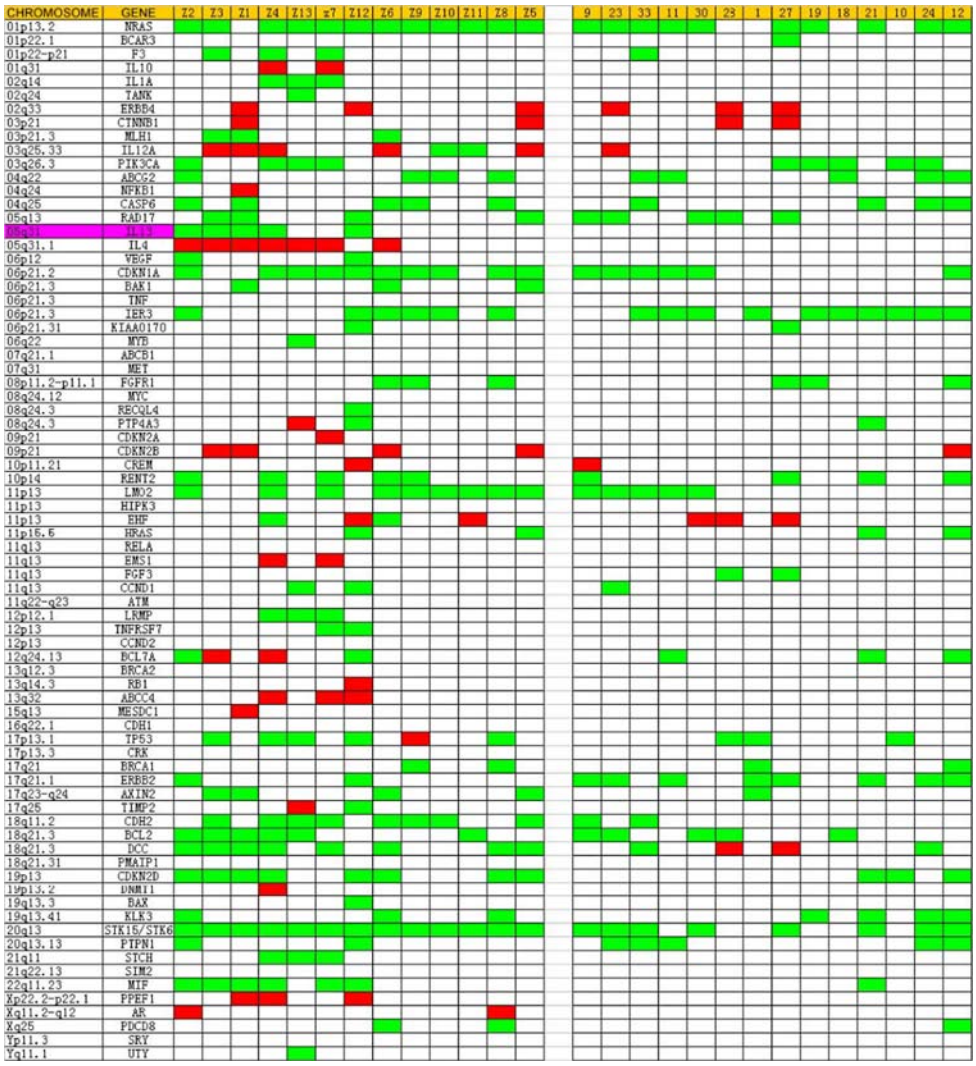

Fig. 1 The copy number changes of all 27 analyzed tumors in a total of 76 genes spanning almost all chromosome arms that were included in the P005 and P006 probe mixes. The left column showed 13 papillary thyroid cancer patients, right column showed 14 thyroid benign nodule ones, the first row indicates patient number. Colors used: *green* gain, *red* loss, and *white* no genetic alteration. IL-13 is emphasized with *purple color*

Table 1 ROC curve analysis of gene copy number changes in PTCs and benign thyroid nodules

Area under ROC curve			Threshold	Sensitivity (%)	1-speciality
Value	Lower limit	Upper limit			
0.868	0.724	1.012	14.5	76.9	0.071

The area under ROC curve was 0.868. The sensitivity of using the threshold value of 14.5 copy number aberrations was 76.9%, and the 1-speciality was 0.071

Several chromosomes (5q, 11p, 18q, 20q, 22q) may contribute to the tumorigenesis of PTCs. The most obvious difference between PTCs and benign nodules was duplication of *IL-13* gene locus on chromosome 5q31. In PTCs, gain of *IL-13* gene was detected in 5 out of the 13 (38%) samples, while none of the 14 samples showed alteration of *IL-13* gene in benign nodules. The results of ROC curve analysis are shown in Table 1. The area under ROC curve is 0.868, which meant moderate accuracy of MLPA analysis in the diagnosis of PTCs. In the present study, the sensitivity of using the threshold value of 14.5 copy number aberrations is 76.9%, and the 1-speciality is 0.071.

Immunohistochemistry

Immunohistochemical analysis was performed to determine the expression of IL-13 protein in thyroid tumors. Positive IL-13 immunoreactivity showed a diffuse intracytoplasmic granular staining (Fig. 2). High IL-13 immunoreactivity was present in PTCs. Forty-seven of 50 PTC samples showed positive IL-13 immunoreactivity, 28 samples were +++, 16 were ++, and 3 were +. In contrast, only 3 of 50 benign tumor samples showed positive IL-13 immunoreactivity, 2 samples were + and 1 was ++ (Table 2). Mann–Whitney *U*-test showed significantly higher IL-13 expression in the PTCs ($P < 0.001$) than in benign nodules. Relatively, normal thyroid tissues surrounding the malignant tumors were negative for IL-13 immunostaining. A few inflammatory cells infiltrating into thyroid were detected through immunostaining of IL-13.

Discussion

The previous CGH studies in thyroid tumors had demonstrated that they had a low prevalence of aberrations, for majority of tumors showing no evidence of chromosomal alteration [13]. However, with the method of MLPA we found that all the samples including PTCs and benign thyroid nodules had genetic aberrations. The method of MLPA could detect more genetic copy numbers alteration than CGH because MLPA had a higher resolution (40 bp) than CGH (10–20 Mb). MLPA was more sensitive to detect the gains or losses of small chromosome parts than CGH [14]. In our present study, the results showed that PTCs had more genetic changes than benign nodules. The difference of genetic copy number alteration was so

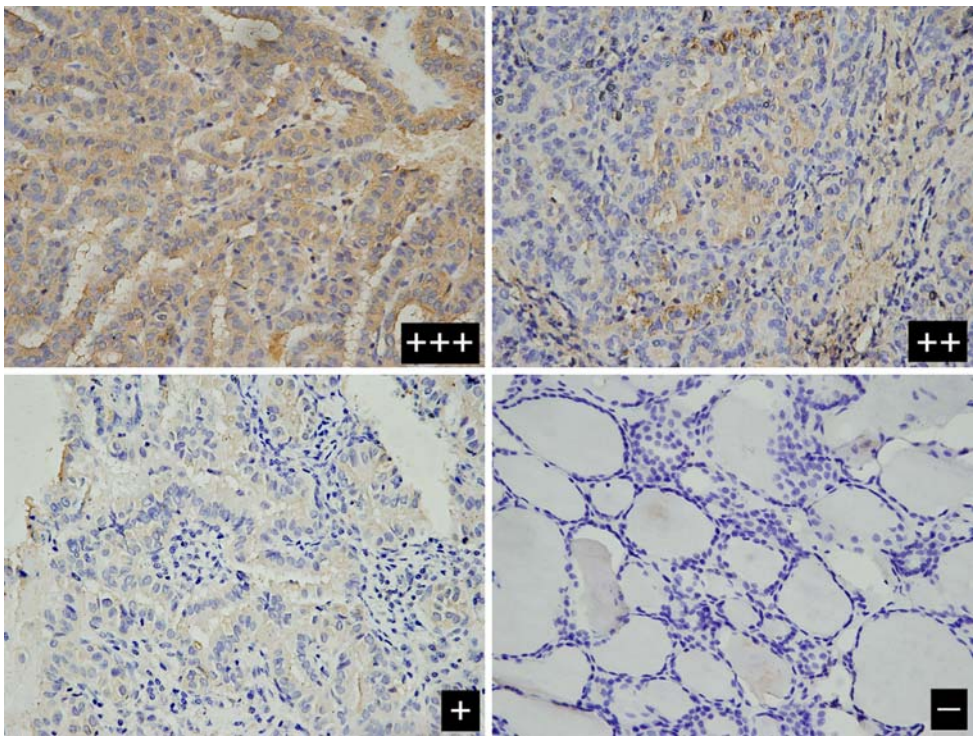

Fig. 2 Immunochemistry result of IL-13 in papillary thyroid carcinomas and benign thyroid nodules labeling index as follows: negative staining cells, index as (−); <30% positive staining cells, index as (+); 30–60% positive staining cells, index as (++); >60% positive staining cells index as (+++). Magnification ×200

Table 2 IL-13 expression in PTCs and benign thyroid nodules by immunohistochemical staining

	PTCs (%)	Benign nodules (%)	*P*
+++	28(56)	0(0)	$P < 0.001$
++	16(32)	1(2)	
+	3(6)	2(4)	
-	3(6)	47(94)	
N	50	50	

+++, >60% positive rate; ++, 30–60% positive rate; +, <30% positive rate; −, negative

significant that it could be used as a genetic diagnostic marker to discriminate between PTCs and thyroid benign nodules. The ROC analysis was the most common method to assess the accuracy of diagnostic tests. The area under the curve was considered as a measure of diagnostic accuracy such that values between 0.5 and 0.7 indicated low accuracy, values between 0.7 and 0.9 indicated moderate accuracy, and values greater than 0.9 indicated high accuracy [18]. In our MLPA results, the ROC curve of 0.868 analysis revealed that genetic copy number alteration could be a good diagnostic marker for discriminating between benign and malignant tumors.

The gain of *IL-13* gene obviously showed the great difference between PTCs and benign nodules. Such aberration was detected in 5 of 13 PTC samples, but in none of the 14 benign nodules. IHC analysis further confirmed that the expression of IL-13 protein in PTCs was much higher than that in the benign ones. There were no studies about the relationship between IL-13 protein and thyroid tumors to be reported. *IL-13* gene polymorphism had been proved to be associated with Graves' disease [19]. Moreover, *IL-13* mRNA could be detected in the majority of GD tissues and primary thyroid cell or HT-ori3 cell after TSH, IL-1, or IFN-γ stimulation. Inversely, unstimulated primary cultures of thyroid cells did not express IL-13 protein [20]. In our data, the protein of IL-13 was expressed strongly in PTCs, weakly in benign nodules, and negatively in normal thyroid tissue around PTCs, which indicated that IL-13 may contribute to the development of PTCs.

IL-13 protein was an immunomodulatory and anti-inflammatory cytokine, usually expressed in activated Th-2 lymphocytes and exerted direct influence on B cells and monocyte/macrophage [21, 22]. Moreover, it modulated a variety of functions in endothelial, mesothelial, and epithelial cells [23, 24]. The IL-13 protein had been detected to be over-expressed in several types of malignant tumor, such as pancreatic cancer and intestinal-type gastric cancer with the significant effects on pathogenesis of carcinomas [25, 26]. The effects of IL-13 protein on various cancer cells were obviously different. Some studies had indicated that IL-13 could enhance the proliferation of some cancer cells, for example IL-13 enhanced pancreatic and prostate cancer cell proliferation [25, 27]. IL-13 was also reported to inhibit apoptosis of HT-29 colon cancer cells in a PI 3-kinase-dependent manner [28]. Moreover, CD44, a factor contributing to the process of carcinogenesis, was induced by up-regulation of IL-13 on human epithelial cell lines of colonic, breast, and liver origin [29]. In other studies, IL-13 was indicated to inhibit the growth of some cancer cells, such as human breast cancer and renal carcinoma cells [30, 31]. The IL-13 protein had revealed the dual characters in pathogenesis of carcinomas. IHC analysis confirmed that there was significant difference in IL-13 expression between malignant and benign thyroid tumors. Therefore, IL-13 may be an important event in the development of PTCs. However, the exact effects of IL-13 on PTCs need further investigation.

The current study showed that MLPA might be an effective method to diagnose PTCs and benign thyroid nodules, and also provided a clue to another relationship between IL-13 and PTCs.

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