Biochemical and Biophysical Research Communications xxx (2016) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

C14orf93 (*RTFC*) is identified as a novel susceptibility gene for familial nonmedullary thyroid cancer

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ARTICLE INFO

Article history:
Received 9 November 2016
Accepted 14 November 2016
Available online xxx

Keywords:
Thyroid cancer
Exome sequencing
C14orf93
RTFC

ABSTRACT

The genetic causes for familial nonmedullary thyroid cancer (FNMTC) remain largely unknown. Through genetic linkage analysis and exome sequencing, *C14orf93* (*RTFC*), *PYGL*, and *BMP4* were identified as susceptibility gene candidates in a FNMTC family. By examining the expression and the oncogenic functions of these candidate genes, *PYGL* and *BMP4* were excluded. We further characterized the functions of the uncharacterized gene *RTFC* in thyroid cancer. *RTFC* promotes thyroid cancer cell survival under starving conditions, and thyroid cancer cell migration. The R115Q, V205M and G209D RTFC mutants enhance the colony forming capacity of thyroid cancer cells, and are able to transform normal thyroid cells. In summary, our data suggest the roles of *RTFC* in thyroid carcinogenesis.

1. Introduction

Nonmedullary thyroid cancer (NMTC) is of follicular cell origin, and accounts for about 95% of all the thyroid cancer cases. The majority of NMTC is sporadic. Yet, it has been reported that approximately 5% of NMTC cases are hereditary, known as familial nonmedullary thyroid cancer (FNMTC) [1,2]. Most of FNMTC is papillary thyroid cancer (PTC), and the inheritance pattern is autosomal dominant with incomplete penetrance. Based on the association with other Mendelian cancer syndromes, FNMTC can be categorized into two types, namely syndromic FNMTC and non-syndromic FNMTC. In syndromic FNMTC patients, thyroid cancer occurs as a minor component of other Mendelian cancer syndromes, such as familial adenomatous polyposis (FAP), Gardner's syndrome, Cowden's disease, Carney's complex type 1, and Werner's syndrome. In contrast, non-syndromic FNMTC is considered to be isolated occurrence of FNMTC [1,3].

The genetic causes for many syndromic FNMTC have been identified. For example, inactivation mutations of APC are causative for FAP and Gardner's syndrome [4,5]. However, the genetic loci for non-syndromic FNMTC are still ambiguous. Through genetic linkage analysis, multiple loci have been identified as FNMTC susceptibility loci. The *MNG1* (multinodular goiter 1) locus on chromosome 14q was the first identified susceptibility locus in a large Canadian family with 18 nontoxic multinodular goiter cases. Yet, further studies revealed that *MNG1* is responsible for only a small fraction of FNMTC [6,7]. The susceptibility loci were mapped to 19p13.2 and 2q21, in a French family with multiple cases of thyroid tumors with cell oxyphilia (TCO) and Tasmanian pedigrees with PTC, respectively [8,9]. Loss of heterozygosity has been detected at 19p13.2 and 2q21 loci in tumors from FMNTC patients, suggesting that these two loci may harbor genes encoding tumor-suppressors [10]. Additional FNMTC susceptibility loci, including 1q21, 6q22, 8p23.1-p22 and 8q24, were identified in other studies [11–14]. It appears that there are many susceptibility loci for FNMTC, and that each susceptibility locus only accounts for certain

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http://dx.doi.org/10.1016/j.bbrc.2016.11.078
0006-291X/

proportion of FNMTC [7].

With many susceptibility loci for FNMTC identified, efforts have been made to search for the corresponding FNMTC susceptibility genes. Two variants (c.925C > A and c.1274G > A) in *TIMM44* in 19p13.2, encoding an inner mitochondrial membrane transporter, were identified to co-segregate with the TCO phenotype. However, no evidence demonstrated that the two variants in *TIMM44* affect the expression or the function of TIMM44. The role of *TIMM44* in FNMTC remains to be proved [15]. Recently, three genes, *SRGAP1*, *FOXE1*, and *HABP2*, have been identified as susceptibility genes for FNMTC [16–19]. Consistent with previous studies on susceptibility loci, multiple susceptibility genes suggest a genetic heterogeneity for FNMTC. Nevertheless, the molecular basis for this disease remains elusive. Efforts are required to identify additional susceptibility genes and elucidate their functions in FNMTC.

2. Materials and methods

2.1. Ethics statement

The institutional review board of Tianjin Medical University Cancer Institute and Hospital (Tianjin, China) approved the study protocols involving human participants (Approval number: bc2012012).

2.2. Subjects

Genomic DNA was extracted from venous blood of affected and unaffected individuals by standard procedures. A total of 9 subjects (I:2, II:1, II:2, II:4, II:6, III:1, III:2, III:3, III:6) in the family were included for linkage analysis. Five individuals (II:1, II:2, II:4 and III:1,III:2) were used for exome sequencing. A cohort of 16 individuals from 14 additional unrelated FNMTC pedigrees and 119 sporadic PTC patients were chosen for further analysis. Analyses also included 518 unaffected individuals of matched geographical ancestry as a healthy control.

2.3. Linkage analysis

Whole-genome genotyping was carried out with the Affymetrix Genome-Wide Human SNP Array 6.0 by CapitalBio Corp. (Beijing, China). Analysis procedures of the SNP data set are described in Supplementary methods.

2.4. Cell culture

BCPAP, K1, 8505c and Nthy-ori 3-1 cells were cultured in growth medium consisting of 90% RPMI-1640 (Gibco), 10% FBS (Hyclone), 2 mM L-glutamine (Invitrogen), 5000 units/ml penicillin and streptomycin (Invitrogen).

2.5. Construction of cell lines

To stably overexpress or knockdown *RTFC* in BCPAP, K1, 8505c, and Nthy-ori 3-1 cells, cells were infected by lentiviruses expressing GFP, WT, V205M, G209D RTFC, or shRNAs targeting *GFP* or *RTFC*. The targeting sequences of shRNAs are shRTFC-1, 5'-GGATGATTACGTGGCCTCTGA-3'; shRTFC-2, 5'-GCTACCACCTGGATGCTAACT-3'; and shGFP, 5'-GAACGGCATCAAGGTGAAC-3'. Forty-eight hours after infection, cells were cultured in medium supplemented with puromycin (1.5–7 μM) for 5–7 days to select for lentivirus infected cells.

2.6. Cell viability assay

Cell viability was measured using MTT (Sangon, China). Cells were harvested from exponential-phase cultures, counted, and plated in 96-well plate at 5×10^3 cells per well in serum-free medium, 6 wells per sample. After starvation in serum-free medium for 5–7 days, cells were incubated with MTT substrate (5 mg/ml) for 4 h. Culture medium was removed, and DMSO was added. Optical density was measured at 560 nm.

2.7. Colony forming assay

For colony forming assay, cells were seeded in a 6-well plate at 300 cells per well in duplicates, and cultured for 10–12 days. Cells were then stained with 0.05% crystal violet before photographing.

2.8. Wound healing assay

Confluent monolayer BCPAP, K1, or 8505c cells were wounded with a 200 μl pipette tip. Cells were washed using PBS to remove cellular debris, and the first images were taken. At a desired timepoint, the second images of the same positions were taken. Migration distance was analyzed using ImageJ software. Four pairs of images were analyzed for each sample.

2.9. Cell transformation assay

Stable GFP, WT, V205M and G209D RTFC overexpression Nthy-ori 3-1 cells were resuspended at a density of 1×10^4 cells/ml in medium containing 0.35% agarose, and then plated onto 6 well plate coated with 0.7% agarose. The cultures were fed once a week, and after 14 days, the colonies were counted.

2.10. Statistical analysis

All data were analyzed by Student's t-test. Statistically significant p values were indicated in figures as follows: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

Additional information of methods is provided in online Supplementary file.

3. Results

3.1. Mapping the FNMTC-linked region

A kindred with five FNMTC patients came to our attention in Tianjin Medical University Cancer Institute and Hospital (Fig. 1A). PTC was confirmed by surgical pathology in four (II2, II4, III1, and III2) of the five patients. They all received total thyroidectomy with central neck dissection. And the remaining one patient I2 was diagnosed as PTC by a fine needle aspiration biopsy. This patient did not receive surgical intervention because of her old age. No other primary cancers have been found in these FNMTC patients and other unaffected family members. The clinical information of these patients was summarized in Table 1.

To identify the disease-causing gene in the FNMTC kindred, whole-genome SNPs of nine family members (five affected and four unaffected, shown in Fig. 1A) were genotyped using the Affymetrix Genome-Wide Human SNP Array 6.0. The accession number for the Whole-genome genotyping data is [GEO]: [GSE75312]. However, no region of high linkage score was identified when the SNP data was analyzed with the software Merlin, likely due to the small number of family members.

Thus, we analyzed the SNP data with a strategy similar to the algorithm used in the CompareLinkage and dChipLinkage software

Please cite this article in press as: C. Liu, et al., *C14orf93* (*RTFC*) is identified as a novel susceptibility gene for familial nonmedullary thyroid cancer, Biochemical and Biophysical Research Communications (2016), http://dx.doi.org/10.1016/j.bbrc.2016.11.078