Aging-associated genes *TNFRSF12A* and *CHI3L1* contribute to thyroid cancer: An evidence for the involvement of hypoxia as a driver

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Abstract. The prevalence of thyroid cancer (TC) is high in the elderly. The present study was based on the hypothesis that genes, which have increased activity with aging, may play a role in the development of TC. A large-scale literature-based data analysis was conducted to explore the genes that are implicated in both TC and aging. Subsequently, a mega-analysis of 16 RNA expression datasets (1,222 samples: 439 healthy controls, and 783 patients with TC) was conducted to test a set of genes associated with aging but not TC. To uncover a possible link between these genes and TC, a functional pathway analysis was conducted, and the results were validated by analysis of gene co-expression. A multiple linear regression (MLR) model was employed to study the possible influence of sample size, population region and study age on the gene expression levels in TC. A total of 262 and 816 genes were identified to have increased activity with aging and TC, respectively; with a significant overlap of 63 genes (P<3.82x10^{-35}). The mega-analysis revealed two aging-associated genes (*CHI3L1* and *TNFRSF12A)* to be significantly associated with TC (P<2.05x10^{-8}), and identified the association with multiple hypoxia-driven pathways through functional pathway analysis, also confirmed by the co-expression analysis. The MLR analysis identified population region as a significant factor contributing to the expression levels of *CHI3L1* and *TNFRSF12A* in TC samples (P<3.24x10^{-4}). The determination of genes that promote aging was warranted due to their possible involvement in TC. The present study suggests *CHI3L1* and *TNFRSF12A* as novel common risk genes associated with both aging and TC.

Introduction

Aging is a fundamental biological process accompanied by alterations in the regulatory activities performed by the endocrine system, including the thyroid gland (1). On the other hand, with aging, the prevalence of hypo- and hyperthyroidism increases (2). Both hypo- and hyperpituitarism are believed to affect longevity (3). Defining a physiological norm for the thyroid hormones is complicated in the elderly by the gradual resetting of the hypothalamic-pituitary-thyroid axis, which leads to increased levels of thyroid stimulating hormone (4).

The prevalence of thyroid neoplasms is increased in the elderly; these tumors are more aggressive in men than in women (5). The mortality rate of thyroid cancer (TC) gradually increases with age, from the ages of 40-45 (6). Notably, young survivors of TC have increased risks for other aging-related diseases, including diabetes, disorders of lipid metabolism, eye disorders, ear conditions and diseases of the musculoskeletal system and connective tissue (7). A majority of TC arise from the epithelial elements of the gland, including thyrocytes and follicular cells. The classification of thyroid tumors into two major groups, differentiated (including papillary, follicular, and medullary) or undifferentiated (anaplastic) carcinoma, based on clinical features and morphology was supported by advances in molecular studies (8).

Importantly, a recent study on DNA methylation and histone modification patterns in >2,000 tumors collected from patients of various ages revealed that most tumor types did not demonstrate age-associated changes in DNA methylation (9), which is in agreement with the theory that the epigenetic clock in cancer cells is reprogrammed (9). Remarkably, this

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theory does not hold for thyroid tumors, which displayed age-associated differential methylation of CpGs (9). In fact, age at diagnosis serves as a strong indicator of prognosis in TC, particularly in well-differentiated tumors (10,11), with an order of magnitude difference in hazard of death from cancer between the youngest and oldest cohorts. It is also of note that thyroid tissue displays the lowest level of DNA methylation changes in cancer and one of the lowest with aging (9).

Overall, previous studies indicate the presence of an association between thyroid carcinogenesis and aging. The present study explored whether genes that promote aging may also play a role in the development of TC, Therefore, besides the identification of previously identified common genes, those that are known to be associated with aging but not with TC are worthy of further study. A mega-analysis of thyroid cancer datasets obtained from Gene Expression Omnibus (GEO) identified two genes, *TNFRSF12A* and *CHI3L1*, as likely contributors to both the process of aging and the thyroid carcinogenesis.

Materials and methods

Study workflow. The workflow was organized as follows. Firstly, the large-scale literature-based mining effort for thyroid cancer (TC)- and aging-associated gene sets was undertaken; these gene sets were compared. For each gene from the list implicated in aging alone, a mega-analysis was conducted in 16 publicly available expression datasets retrieved from GEO. For genes that showed significant change in expression across analyzed datasets, functional pathway analysis and protein-protein interaction (PPI) by co-expression analysis were conducted, then conclusions on their pathogenic significance in TC were made.

Literature-based relation data. Relation data for both aging and TC were extracted and analyzed using Pathway Studio (www.pathwaystudio.com), and the results were downloaded into a genetic database Aging_TC, hosted at http://database.gousinfo.com. The downloadable format of the database was available at http://gousinfo.com/database/Data_Genetic/Aging_TC.xlsx, with different section of the results presented in different worksheets (e.g., age-specific genes were presented in worseheet 'Aging_alone genes'). In this study, we refered to these worksheets in the form of Aging_TC→'worksheet name'. Beside the list of analyzed genes (Aging_TC→Aging_alone genes, Aging_TC→TC_alone genes, and Aging_TC→Common genes), supporting references for each disease-gene relation are presented (Aging_TC→Ref for Aging_alone genes, Aging_TC→Ref for TC_alone genes, and Aging_TC→Ref for Common genes), including titles of the references and the sentences describing identified disease-gene relationships. The information could be used to locate a detailed description of an association of a candidate gene with aging and/or TC. Please refer to Aging_TC→DataNote for the decryption of each worksheet.

Data selection for mega-analysis. The relevant expression datasets available at GEO (https://www.ncbi.nlm.nih.gov/geo/) were retrieved with the keyword 'thyroid cancer' (n=91). The following criteria were applied: i) Organism, *Homo sapiens*; ii) data type, RNA expression; iii) sample

size, ≥10; and iv) the studies were performed according to case-control design. A total of 16 datasets remained available for the mega-analysis.

Mega-analysis models. To discern the effect sizes of the selected genes in a case vs. control expression comparison, both fixed-effect and random-effects models were employed. The expression log fold change (LFC) was used as the effect size. The results derived from both models were compared. In order to assess the variance within and between different studies, the heterogeneity of the mega-analysis was analyzed. In the case that total variance Q was equal to or smaller than the expected between-study variance df, the statistic $ISq=100\% \times (Q-df)/Q$ was set as 0, and a fixed-effect model was selected for the mega-analysis. Otherwise, a random-effects model was selected. The genes with significant effects were identified according to the following criteria: $P<1\times10^{-7}$ and effect size (LFC) >1 or <-1.

Multiple linear regression analysis. A multiple linear regression (MLR) model was employed to study the possible influence of three factors on the changes in gene expression in TC: Sample size, population region, and study age. P-values and 95% confidence interval (CI) were reported for each of the factors.

Pathway analysis. For the set of genes identified through expression mega-analysis described above, a functional pathway analysis was conducted with an aim to identify potential biological associations between the selected genes and the TC. The analysis was performed using the 'Shortest Path' module of Pathway Studio (www.pathwaystudio.com).

Co-expression analysis. For each pair of the genes/proteins identified in the pathway analysis, a study of their co-expression was executed. The purpose of this analysis was to identify possible protein-protein interaction (PPI) of the proteins involved. The Fisher's Z was employed as the effect size for the mega-analysis, as shown in the equation below:

$$\text{Fisher } Z = 0.5 \times \log\left(\frac{1+Correlation}{1-Correlation}\right) \qquad (1)$$

All co-expression associations with P-value $<1\times10^{-4}$ and Fisher's Z-value ≥0.3 or ≤-0.3 were identified as significant, and then presented as a Cytoscape-imaged PPI network.

Results

Genes commonly affected by the process of aging and by the carcinogenesis in the thyroid gland. The curated Aging_TC database identified 262 genes with substantially increased expression or activity with aging (supported by 1,495 scientific references) and 816 genes associated with the pathogenesis of TC (supported by 4,169 references). A total of 63 genes were identified to be involved in both aging and TC (right tail Fisher's exact test, $P=3.82\times10^{-35}$; Fig. S1), which accounts for about a quarter of the genes associated with aging (24.05%). The descriptions of these 63 genes are presented in Table I. Further information on these genes was also provided in

Table I. Common genes associated with aging and thyroid cancer.

Name	Entrez Gene ID	Human chromosome position
SERPINC1	462;304917;11905	1q25.1
IL17A	16171;301289;3605	6p12.2;6p12
EDN1	1906;24323;13614	6p24.1
LCN2	16819;170496;3934	9q34.11;9q34
PTGS2	19225;29527;5743	1q31.1;1q25.2-q25.3
HLA-G	24747;14991;3135	6p21.3;6p22.1
CSF1	1435;12977;78965	1p13.3
MIF	4282;17319;81683	22q11.23
PTH	19226;24694;5741	11p15.3;11p15.3-p15.1
CYP27B1	1594;13115;114700	12q14.1
CYP24A1	25279;1591;13081	20q13;20q13.2
HUWE1	59026;10075	Xp11.22
EIF2AK2	5610;54287;19106	2p22-p21;2p22.2
PDGFRB	5159;24629;18596	5q32;5q33.1
APP	54226;351;11820	21q21.3
HYOU1	10525;12282;192235	11q23.1-q23.3;11q23.3
EGF	25313;1950;13645	4q25
TNF	24835;21926;7124	6p21.3;6p21.33
HMOX1	15368;3162;24451	22q12.3;22q13.1
MIR21	406991	17q23.1
MMP9	81687;17395;4318	20q11.2-q13.1;20q13.12
FAS	246097;14102;355	10q23.31;10q24.1
DPP4	25253;1803;13482	2q24.3;2q24.2
KLK3	18048;18050;354;13648;16619;16618;16613;16624;16612;16623;16622; 13646;16617;16616;16615	19q13.33;19q13.41
PROS1	19128;81750;5627	3q11.2;3q11.1
FASLG	14103;356;25385	1q23;1q24.3
GPX3	2878;64317;14778	5q23;5q33.1
ALB	11657;213;24186	4q13.3
DCN	1634;13179;29139	12q21.33
B3GAT1	27087;76898;117108;964	11q25
ADIPOQ	9370;246253;11450	3q27;3q27.3
ARG2	11847;29215;384	14q24.1
RUNX3	12399;156726;864	1p36.11;1p36
PRDX1	5052;18477;100363379;117254	1p34.1
HTRA1	5654;65164;56213	10q26.13;10q26.3
RCAN1	54720;1827	21q22.12
CTNNB1	84353;12387;1499	3p21;3p22.1
BAG3	9531;29810;293524	10q25.2-q26.2;10q26.11
TGFB1	21803;7040;59086	19q13.1;19q13.2
TGFA	24827;21802;7039	2p13;2p13.3
CTSD	1509;171293;13033	11p15.5
MUC1	4169;4582	1q21;1q22
TRAP1	68015;10131;287069	16p13.3
CDKN1A	12575;114851;1026	6p21.2
CCNA2	12428;114494;890	4q27
CCR2	1231;12772;729230	3p21.31
IL21	59067;365769;60505	4q26-q27;4q27
BAX	12028;24887;581	19q13.33;19q13.3-q13.4
IL4	287287;3565;16189	5q31.1
ENO1	2023;24333;433182;13806	1p36.23;1p36.2
HGF	3082;15234;24446	7q21.11;7q21.1

Table I. Continued.

Name	Entrez Gene ID	Human chromosome position
TNFSF10	246775;22035;8743	3q26.31;3q26
IL6	24498;3569;16193	7p15.3;7p21
THRA	7067;81812;21833	17q11.2;17q21.1
ANGPT2	89805;11601;285	8p23.1
EP300	170915;2033;328572	22q13.2
GSTM1	2944;14863;24424	1p13.3
CCL2	287562;6347;20293	17q12;17q11.2-q12
TXNIP	117514;56338;10628	1q21.1
GSTT1	2952	22q11.23
POSTN	50706;361945;10631	13q13.3
NQO1	18104;1728;24314	16q22.1
IL10	16153;25325;3586	1q31-q32;1q32.1

Aging_TC→Common genes and Aging_TC→Ref for common genes.

In order to assess the functional profile of the 63 genes associated with both aging and TC, a gene set enrichment analysis (GSEA) against the GO and Pathway Studio Ontology was conducted. The GSEA showed that these common genes were mainly involved in the protein kinase domain, cell proliferation, tissue development, gland development and response to hormone processes. Specifically, this analysis uncovered a total of three pathways/gene sets associated with cell apoptosis (26 unique genes) and 2 pathways/gene sets associated with cell growth proliferation (25 unique genes). A bar plot of the 39 pathways and enriched genes out of the 63 common genes are presented in Fig. S2.

Gene expression analysis result. Although there was a significant overlap between aging- and TC-associated gene sets (n=63; P=3.82x10^{-35}), a majority of the aging-associated genes (n=199 or 75.95%) have not been yet implicated in the pathogenesis of TC. Therefore, the association between each of these 199 genes with TC was examined, using 16 gene expression datasets shown in Table II (12-26). The detailed description of the results is presented in Aging_TC→Mega-analysis. The significance of association criteria (P<1x10^{-7} and absolute LFC>1) was met by two genes and presented in Table III.

For each gene, a LFC was estimated from the majority of the 16 studies (a total of 16 and 14 studies for *CHI3L1* and *TNFRSF12A*, respectively). As shown in Fig. 1, the mRNA expression levels of *TNFRSF12A* demonstrated strong between-study variances (ISq=54.07% and Q test P=8.2x10^{-3}); therefore, the random-effects model was selected for the mega-analysis. In contrast, no significant between-study variance was observed for the gene *CHI3L1* (ISq=14.73% and Q test P>0.28); therefore, the fixed-effect model was selected for analysis of its mRNA expression levels. This identified the sample population region (country) as a significant factor that influences the LFC of both genes in the case of TC (P<3.24x10^{-4}; Table III).

Functional pathway analysis. According to the *de novo* approach selected for the identification of novel TC-associated

genes, no prior direct link to the pathogenesis of TC were known. However, Pathway Studio-guided 'shortest path analysis' revealed plausible associations of *CHI3L1* and *TNFRSF12A* genes with TC, with a set of common interactions (Fig. 2A).

Using Pathway Studio, the 'shortest path' analysis was conducted to identify associated genes that link *CHI3L1* and *TNFRSF12A*-encoded molecules to the pathogenesis of TC in a unidirectional way. For example, the association between *CHI3L1* and transforming growth factor β1 (TGFB1) in TC was identified. YKL-40, also known as chitinase-3-like protein 1 (*CHI3L1*), is a secreted glycoprotein that binds to interleukin-13 receptor α2 (IL-13Rα2) and subsequently stimulates the production of TGF-β1 (27), a key molecule in thyroid carcinogenesis and considered as a new prognostic and therapeutic target for TC (28,29). The details for all other associations are presented in Fig. 2A, and are described in Aging_TC→TC-2Genes_potential pathways. This reference information included the type of association, the amount of underlying supporting references, and the relevant sentences where these relationships were identified and described. The shortest pathway analysis was conducted using the Pathway Studio (www.pathwaystudio.com). All TC-associated genes reported in the shortest pathway analysis were employed in the identification of the TC-associated genes.

In order to confirm the associations depicted in Fig. 2A, another mega-analysis was conducted using 16 datasets, with the purpose to evaluate the co-expression pattern of mRNAs encoded by *CHI3L1* and *TNFRSF12A*, and 31 genes that link the two genes with TC. The association between *CHI3L1* and *TNFRSF12A* and 13 of the 31 genes presented in Fig. 2A was validated (P<0.005; Fig. 2B). Please refer to Aging_TC→MetaResults_ShortestPath for the detailed description of the associations presented in Fig. 2B. In addition, a PPI network connecting the products of *CHI3L1* and *TNFRSF12A* and the 'bridge' genes (n=31) were generated using Cytoscape (Fig. 3). The detailed information of the mega-analysis for the co-expression was presented in Aging-TC→PPI, including a list of the genes (nodes) of the PPI network, and the mega-analysis results for each pair of gene-gene correlation.

Table II. Datasets used for thyroid cancer-aging relation mega-analysis.

Study	Dataset GEO ID	Control, n	Case, n	Country	Refs.
Jarzab et al, 2015	GSE35570	51	65	Poland	(12)
Rusinek et al, 2015	GSE58545	18	27	Poland	(13)
Swierniak et al, 2015	GSE58689	18	27	Poland	(13)
Tarabichi et al, 2015	GSE60542	34	33	Belgium	(14)
von Roemeling et al, 2015	GSE65144	13	12	USA	(15)
Versteyhe et al, 2013	GSE39156	16	48	Belgium	(16)
Pita et al, 2013	GSE53157	3	24	Portugal	(17)
Tomas et al, 2012	GSE29265	20	29	Belgium	N/A
Tomas et al, 2012	GSE33630	45	60	Belgium	(18,19)
Giordano et al, 2011	GSE27155	4	95	USA	(20,21)
Yu et al, 2008	GSE5364	58	270	Singapore	(22)
Fontaine et al, 2007	GSE6339	135	48	France	(23)
Salvatore et al, 2007	GSE9115	4	15	USA	(24)
Reyes et al, 2006	GSE3678	7	7	USA	N/A
Vasko et al, 2006	GSE6004	4	14	USA	(25)
Liyanarachchi et al, 2005	GSE3467	9	9	USA	(26)

N/A, not applicable.

Table III. Significant genes from mega-analysis [$P<1x10^{-7}$ and abs(LFC)>1] involved in aging and thyroid cancer.

Gene name	Mega-analysis results					Multiple linear regression analysis for three factors		
	Random effects model	Datasets included	LFC	STD of LFC	P-value	Sample size	Population region	Year of study
CHI3L1	0	16	2.88	0.42	$5.10x10^{-12}$	0.24	$2.77x10^{-4}$	0.15
TNFRSF12A	1	14	1.79	0.33	$2.05x10^{-8}$	0.35	$3.24x10^{-4}$	0.56

LFC, log fold change. STD, standard deviation.

Discussion

The present study aimed to identify novel molecular pathways, which connect the process of aging and the development of TC. By removing all known associations between curated sets of genes involved in aging and TC, uncovered aging-associated contributors to TC were identified. According to the pre-selected significance of association criteria ($P<1x10^{-7}$ and abs(LFC)>1), two aging-associated genes, TNFRSF12A and CHI3L1, were found to be involved in the development of TC.

The role of TNFRSF12A and CHI3L1 in the aging-associated disease is well described, whereas no apparent connections to the malignant processes in the thyroid were reported thus far. TNFRSF12A encodes for an exclusive receptor for tumor necrosis factor-related weak inducer of apoptosis (TWEAK), an interacting pair of molecules involved in age-associated pathological changes in skeletal muscle and other organs (30-32). CHI3L1 encodes for YKL-40, a glycoprotein upregulated in a variety of inflammatory conditions commonly found in the elderly, including chronic obstructive pulmonary disease and neurodegenerative diseases (33,34).

Employing a Pathway Studio-guided 'shortest path analysis' revealed plausible associations of TNFRSF12A and CHI3L1 with TC, simultaneously highlighting a set of common interactors that included the signaling molecules AKT1, RAC1, MAPK1 and the soluble proteins TNF-α, albumin, TIMP1 and MMP9.

Among these, the association of TNFRSF12A and CHI3L1 with AKT1 was notable, as this molecule was both overexpressed and over-activated in TC (35). Moreover, increased Akt signaling led to increased Bcl-2 promoter activity and cell survival (36). The interaction between TNFRSF12A and TWEAK also positively regulated pro-survival molecules
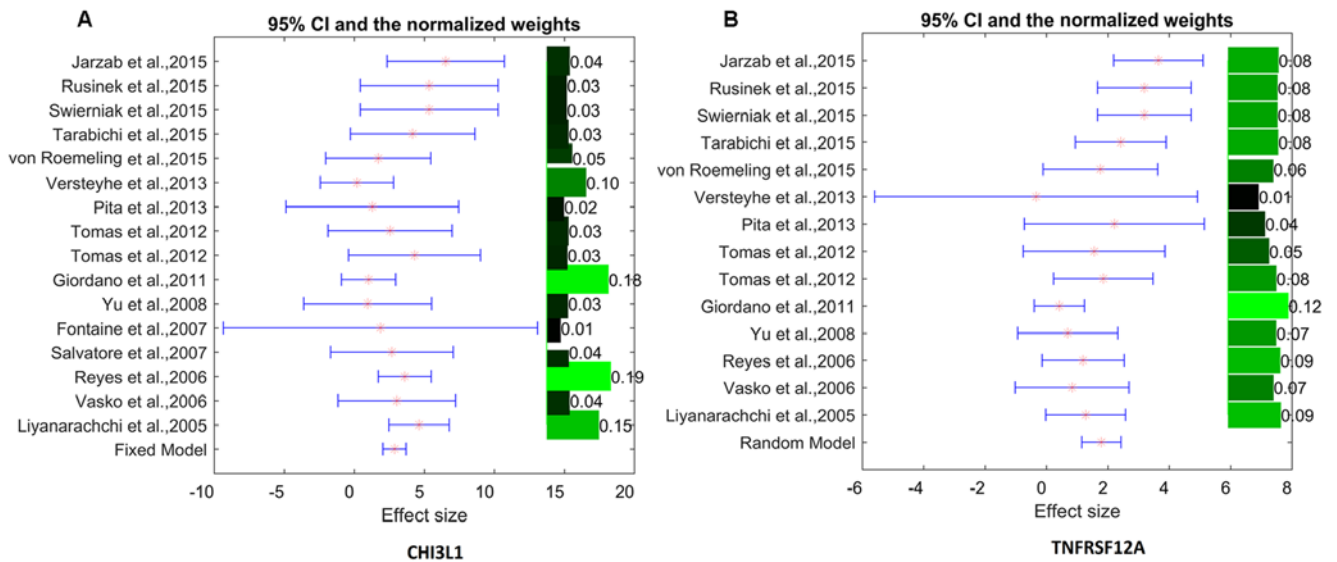
Figure 1. Effect size, 95% CI and weights of the expression levels of *TNFRSF12A* and *CHI3L1*. (A) Mega-analysis results for *CHI3L1*; (B) Mega-analysis results for *TNFRSF12A*. Fixed-effect model and random-effects model were employed for *CHI3L1* and *TNFRSF12A*, respectively. CI, confidence interval.
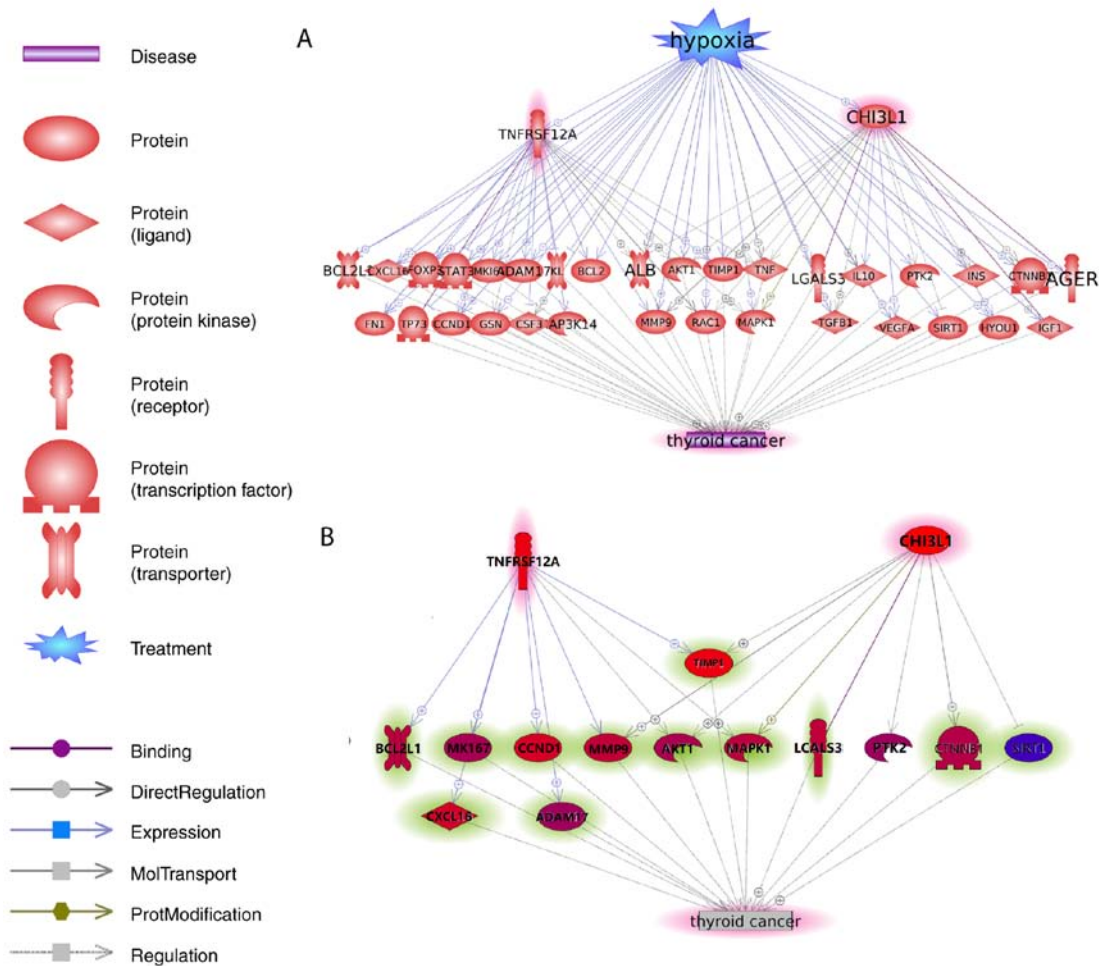

Figure 2. Hypoxia driven interaction network that links *CHI3L1* and *TNFRSF12A* to thyroid cancer. (A) The network generated in Pathway Studio environment (www.pathwaystudio.com). Each relation (edge) in the figure has one or more supporting references. (B) Network containing only protein-protein interactions validated by 14 thyroid cancer expression datasets.

of the *Bcl2* family (37,38), and, therefore augmented the pro-survival signal of *AKT1* (39).

Among the soluble molecules, angiogenic matrix metalloproteinase (*MMP)9* was found to be associated with
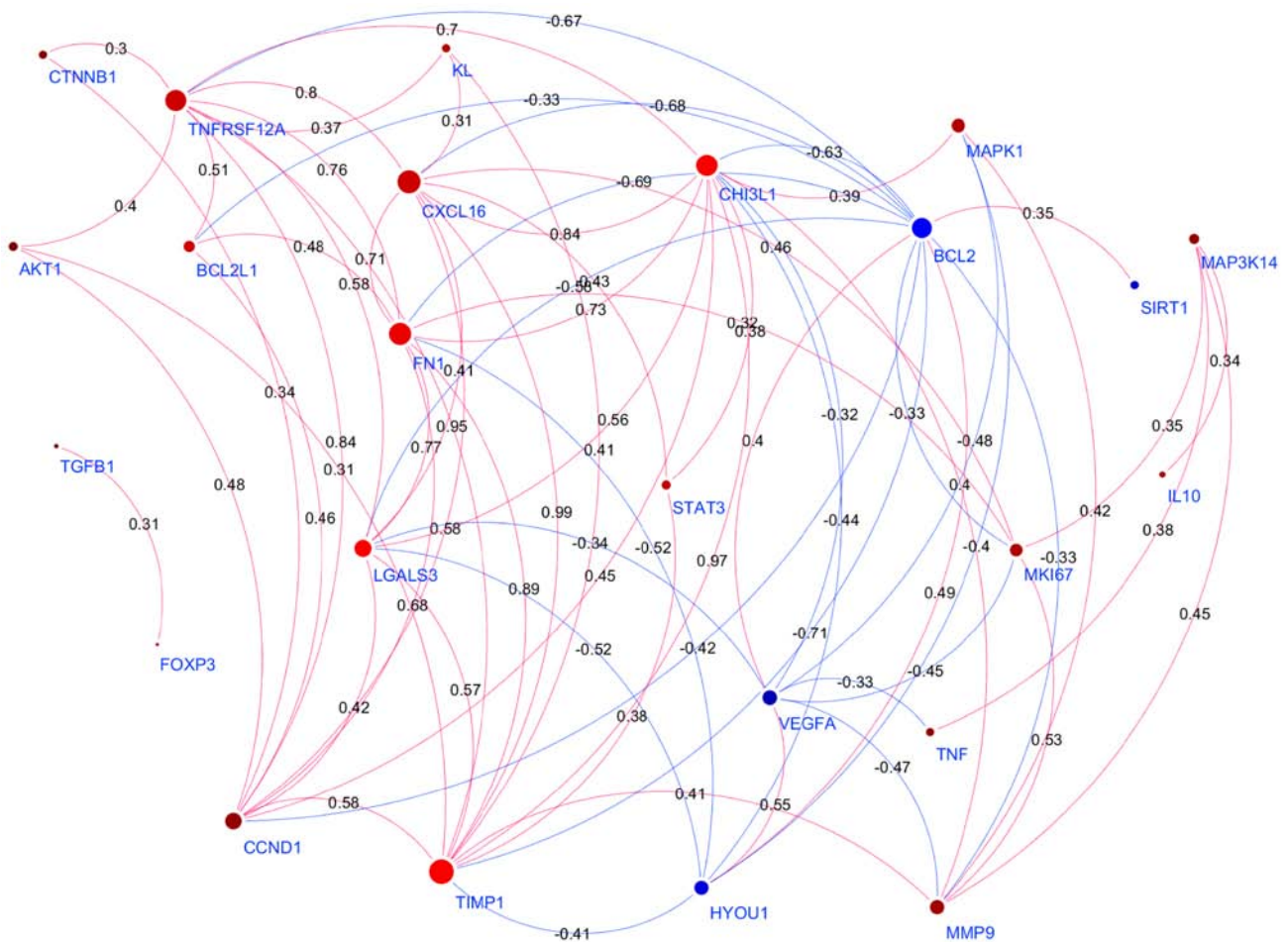
Figure 3. Protein-protein interaction network generated in Cytoscape for *CHI3L1* and TNFRSF12A and the 'bridge' genes. Each edge represents a significant association ($P<1\times10^{-4}$) between the mRNA expression levels encoded by *CHI3L1* and *TNFRSF12A*. Positive associations are highlighted in red; negative associations are highlighted in blue. A red node represents an overall positive association within the network, whereas a blue node represents an overall negative association. The size of the node represents the centrality of the respective protein. The larger the size, the higher the centrality of the protein. The labels on the edge represent the correction coefficients in terms of Fisher's Z-value as derived from the mega-analysis.

TNFRSF12A and *CHI3L1*, which participates in follicular TC cell invasion (40). *TIMP-1*, acts as an inhibitor for *MMP9* expression that is often co-expressed with this molecule in thyroid tumors and serves as a reliable surrogate marker for BRAF-mutated status and likely aggressiveness (41). Notably, *TIMP*-1 serves as a prominent PPI hub gene in the Cytoscape network built upon co-expression of *TNFRSF12A* and *CHI3L1*, along with *LGALS3*, a specific biomarker of well-differentiated thyroid carcinomas (42), *CXCL16*, which mediated the involvement of macrophages in the invasion of papillary TCs (43) and fibronectin, an EMT biomarker which promoted migration and invasion of papillary thyroid cancers (44).

Importantly, many of the molecules discussed above were upregulated in response to hypoxia (45), or stimulated the expression of key mediators of the antihypoxic response (46), or both (47,48). Both *HIF-1α* and *HIF-2α* were reported to be expressed in TC (49). Moreover, a growing evidence points out that hypoxia plays a significant role in the maintenance of thyroid cancer stem cells (CSC) (50). General hypoxia due to diminished vascularization is a well-known characteristic of aging tissues, possibly due to endothelial dysfunction as well decreased function of endothelial progenitor cell function (51,52). For more information regarding the hypoxia-gene

regulation presented in Fig. 2A, please refer to Aging_TC→TC_2Genes_potential pathway. Therefore, it can be speculated that the age-dependent increase in hypoxia may also contribute to the increased aggressiveness of TC observed in the elderly (10,11).

The results could be influenced by multiple factors, including sample size and sample source. Considering the fact that the pathology of disease could change with time, the publication date/age was checked in the present study. The MLR analysis showed that country region was a significant factor that could influence the gene expression levels of TC genes, but not the other two factors. Other influencing factors of TC gene activities could include mortality or epigenetics. However, due to be the limitation of the metadata, these factors were not amiable for analysis in the datasets employed in the present study, which would be valuable for future studies.

In conclusion, the present study conducted a functional pathway and co-expression analysis, and mined a set of genes associated with aging. *TNFRSF12A* and *CHI3L1* were identified as previously unrecognized contributors to the development of thyroid tumors, which are known for unusual increase in aggressiveness in the elderly. An analysis of the

Cytoscape network built upon co-expression of *TNFRSF12A* and *CHI3L1* points towards tissue hypoxia as a bridging factor, which is common for the pathophysiology of aging and the development of TC.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ML, HC, AB and QS developed the study design, analyzed the data, and wrote the original manuscript. KCK, LH, SH and JF contributed to data analysis and manuscript writing and revision. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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