FUCA1 is induced by wild-type *p53* and expressed at different levels in thyroid cancers depending on *p53* status

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Abstract. Fucose residues of cell surface glycans, which play important roles in growth, invasion and metastasis, are added by fucosyltransferases (FUTs) and removed by α-L-fucosidases (FUCAs). By the differential display method, we isolated a 3' non-coding region of *α-L-fucosidase-1 (FUCA1)* (a gene coding for the lysosomal fucosidase-1 enzyme) as a wild-type p53-inducible gene: 18S and 20S *FUCA1* mRNA species were induced in Saos-2 cells transfected with a temperature-sensitive p53 mutant at the permissive temperature. By microarray analyses of thyroid cancer biopsy samples, *FUCA1* RNA expression levels were found to be lower in anaplastic thyroid cancer samples (ATCs), while they were higher in papillary thyroid cancer samples (PTCs) and in normal thyroid tissues. Since most ATCs were reported to carry the mutated form of *p53*, while PTCs carry mostly the wild-type form of *p53*, it is likely that FUCA1 expression levels are regulated, at least in part, by the *p53* status in thyroid cancers. In order to better understand the role played by *FUCA* genes in thyroid tumorigenesis, we examined the clonogenic potential *in vitro* of thyroid cell lines transfected with either *FUCA1* or *FUCA2* (the latter gene coding for a secreted, non-lysosomal enzyme). We found that α-L-fucosidases did not suppress grossly cell growth. Contrary to what we observed with the expression of *FUCA1*, the *FUT8* expression levels were found high in ATCs but lower in PTCs and normal thyroid tissues. Taken together, these results suggest the possibility that the higher fucose levels on cell surface glycans of aggressive ATCs, compared to those of less aggressive PTCs, may be at least in part responsible for the more aggressive and metastatic phenotype of ATCs compared to PTCs, as the expression levels of *FUCA1* and *FUT8* were inversely related in these two types of cancers.

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Introduction

Glycans differentially modified with fucose residues consist of glycosylated proteins or lipids. Glycoproteins on the cell surface play important roles in the interaction with other cell types, extracellular matrices or with pathogenic microorganisms, and in the binding of growth factors on the cell surface. Fucose moieties of glycans are removed by α-L-fucosidases (FUCA)1 and 2 and added by fucosyl transferases (FUT1-11), thereby modulating these interactions (1-4). Cell to cell interactions are essential phenomena in the metastatic process of cancer cells, which involves dislodging of cancer cells from a primary tumor site to target organs through intravasation and extravasation, eventually leading to the establishment of metastatic foci (5,6). It has been reported previously that α-L-fucosidase activity may prevent cancer progression, since *in vitro* removal of α-L-fucose residues from glycoproteins present on the surface of breast cancer cells with the bovine α-L-fucosidase enzyme, modulated the adhesion-motility of a breast cancer cell line, as examined in *in vitro* models (7,8). On the other hand, core-fucosylation by FUT8 has been reported to be crucial for EGF binding and the subsequent EGFR activation by dimerization, followed by phosphorylation (9). Thus, cell surface fucose residues play important roles in cancer cell growth and progression (2).

p53 is a transcription factor that activates genes involved in cell growth regulation (10). We have previously constructed *in vitro* a human temperature-sensitive *(ts)* mutant substituting alanine for valine at codon 138 of the *p53* (11).

When this mutant gene was expressed in p53-negative Saos-2 osteosarcoma cells, the *ts-p53* construct induced growth arrest

in Saos-2 cells by transactivating p21 CDK2 inhibitor at the permissive temperature (32.5˚C) (12). By differential display analysis of Saos-2 cells expressing this *ts-p53* mutant, in the present study we identified human *FUCA1* as a p53 target gene. Further, we analyzed *FUCA1* expression in thyroid cancers and thyroid cancer-derived cell lines. We chose two different histotypes of thyroid cancers, since the majority of anaplastic thyroid cancers (ATCs) usually carry mutated *p53*, while less aggressive, papillary thyroid cancers (PTCs) carry mostly the wild-type *p53* (13). Since it has been previously shown that fucosylation of cell surface glycans plays an important role in the metastatic spread of thyroid cancer (14), we examined the expression levels of both *FUCA1* and *FUT8* in the same set of tissue microarrays consisting of samples of ATCs, PTCs and normal thyroid tissues. The results reported here strongly suggest that the differences of *p53* gene status in the two different sets of thyroid cancers examined and the differences in the expression levels of the two above-mentioned genes may be responsible for modulating the cell surface fucosylation in the two types of thyroid cancers and may contribute to their different clinical behavior. Preliminary results concerning the observation that *FUCA1* is a *p53*-target gene were presented in the 16th International Symposium on Molecular Medicine in Crete, Greece, 2013 (15). During the preparation of this study, Ezawa *et al* (16) and Baudot *et al* (17) reported that FUCA1 is a downstream target of p53.

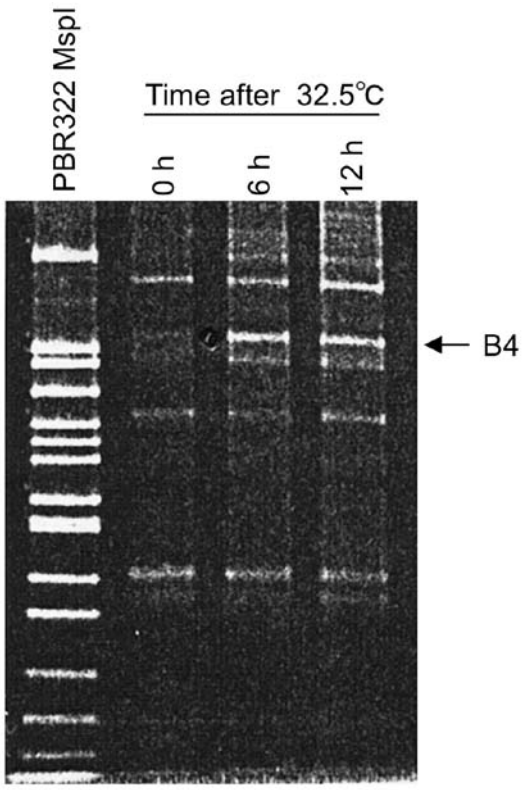

Figure 1. Differential display of p53-induced DNA fragments. cDNA prepared from RNAs of S/ts-p53 6 and 12 h after incubation at 32.5˚C were PCR-amplified with B4 5' arbitrary primer (5'-CGTCTTTCTG-3') and 3' anchor primer (5'-GT15G-3'). Portions of PCR products were run on 12% PAGE, stained with ethidium bromide. The arrow indicates the B4 fragment.

Materials and methods

Cells and culture. Human osteosarcoma cells expressing exogenously a *temperature-sensitive (ts) p53-alanine138valine (ts-p53A138V)* mutant gene (S/ts-p53) and the corresponding cell clones transfected with empty vector (S/neo) were previously described (12). When the cells were cultured in Dulbecco's modified MEM medium with 10% fetal bovine serum (FBS), the growth was arrested in G1 or G2/M phase at 32.5˚C. The normal human thyroid cell line NTHY, consists of normal thyroid cells immortalized with SV40 ori-DNA (18), TPC1 is a cell line established from a papillary thyroid cancer (PTC) (19) and CAL62 is a cell line (20) established from a human anaplastic thyroid cancer (ATC). These cells were grown in RPMI-1640 (Gibco/Invitrogen; Thermo Fisher Scientific Co., Tokyo, Japan) containing 10% FBS.

Preparation of cell RNA. Cellular RNAs were prepared from cell clones of S/*ts-p53*, which had been cultured at 37.5˚C and then shifted to 32.5˚C for various lengths of time. RNAs were prepared, using an Isogen kit (Nippon Gene, Toyama, Japan), as described in the kit instructions (12).

Differential display. An mRNA fingerprinting kit from Nippon Gene was used. Total cell RNA prepared was further purified by treating with RNase-free DNase (Nippon Gene), as described in the kit instructions. cDNAs corresponding to 3' terminal regions of mRNAs were amplified by PCR, using one of 3 different anchor primers (GT15G, CT15G, and AT15G), and one of 50 different 5' arbitrary primers, included in the kit. After PCR amplification, portions were run on a 12% polyacrylamide gel (PAGE) with MspI-digested pBR322 DNA fragments (New England Biolabs, Ipswich, MA, USA) as size markers. DNA fragments were visualized under a 300 nm UV light after staining with ethidium bromide (0.5 μg/ml). A band, the intensity of which showed an increment after temperature shift-down, was excised from the gel and the DNA present in the band (B4 fragment) was extracted, purified, and ligated to a T/A cloning vector, pCR2.1 (Invitrogen Life Technologies, Tokyo, Japan) (21). The insert of pCR2.1 vector was sequenced with M13 forward and reverse primers as per the instructions provided in the Invitrogen manual.

Northern hybridization. Northern hybridization was performed as previously described (12). After the transfer of RNA to a nylon membrane filter (Millipore Co., Tokyo, Japan), it was exposed to a UV light. During this fixation, integrity and equal loading of RNAs were confirmed (data not shown). ^{32}P-dCTP-labeled DNA probe was prepared from the cloned B4 fragment, and used to hybridize Northern filters. The hybridized filters were washed, and exposed to an image plate, which was analyzed by a Fuji image analyzer (Fuji Film Co., Tokyo, Japan) (12).

Western blot analysis. Cellular proteins were prepared from cells with or without the temperature shift-down, by lysing on plates, with RIPA buffer (Nacalai Tesk, Kyoto, Japan), as previously described (22). The protein lysates were run on a 5-20% precast polyacrylamide gel (Ato-kabushikieyashiro Inc., Tokyo, Japan) and the proteins were transferred to a membrane filter (Immobilon, Millipore Co.). For the integ-

Figure 2. Blast search for B4 DNA sequence. The upper and lower sequences indicate the B4 clone and *FUCA1* cDNA, respectively. Sequence derived from the B4 5' anchore primer is boxed. poly(A) addition signal sequence is marked in red.

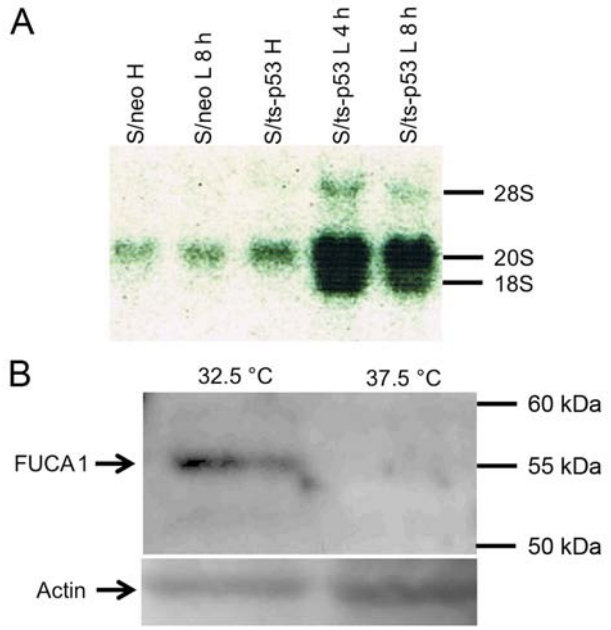

Figure 3. Induction of *FUCA1* RNA by Northern blot analysis (A) and FUCA1 protein by western blotting, in S/ts-p53 cells after incubation at 32.5˚C (B). The lower panel shows actin band around 42 kD stained by Ponceau S.

rity and equal loading of protein lysates, membranes were stained with Ponceau S and destained as described (23). The membranes were then blocked with 5% skim milk and probed with anti-FUCA1 antibody (AV54293, polyclonal antibody made with an immunogen consisting of FUCA1 amino acids 144-193, Sigma-Aldrich, Tokyo, Japan). The filter was incubated with a secondary anti-rabbit antibody conjugated with peroxidase, and the protein bands were visualized by the use of an ECL illumination kit (Amersham, Tokyo Japan), as previously described (22). The gel images were captured by a LAS-4000 image analyzer (GE Healthcare, Tokyo, Japan).

Microarray analysis of FUCA1 and FUT8 gene expression in thyroid cancer tissues. Tumor and normal tissues from thyroid cancer patients were collected. RNA preparation from tissues, cDNA synthesis, hybridization to microarray plates, data collection, and data analyses have been previously described (24). The data collected by cDNA microarray analyses were used to determine the expression levels of *FUCA1* and *FUT8* gene in 8, 4, and 4 biopsy samples derived from PTC, ATC, and normal tissues, respectively.

Colony forming efficiency of thyroid cells. DDK-MYC-tagged *FUCA1* DNA, DDK-MYC-tagged *FUCA2* DNA, and the empty vector DNA, all carrying the neomycin resistance gene (OriGene, Rockville, MD, USA) or H_2O instead of plasmid DNA, were transfected with a transfection kit (Roche Diagnostics, Germany) in Opti-MEM (Gibco/Invitrogen; Thermo Fisher Scientific Co.) to 70% confluent thyroid normal or cancer cells grown in 60-mm plates. The next day the medium was replaced with Dulbecco's modified MEM containing 10% FBS and 600 μg/ml G418 (Invitrogen; Thermo Fisher Scientific Co.). Thereafter, the medium was changed twice a week and colonies appearing 4 weeks later were stained with Giemsa. Colony formation of thyroid normal or cancer cells were compared between cells transfected with *FUCA1* or *2* plasmid DNA or with empty plasmid vector.

Results

Differential display to identify p53-target gene. To search for *p53* target genes by differential display, we took advantage of the use of S/ts-p53 cells that expressed a *ts-p53* mutant gene *(ts-p53A138V)*. Total cellular RNAs from S/ts-p53 cells were prepared at different times after temperature shift-down to the permissive temperature (32.5˚C). When B4 was used as a 5' arbitrary primer (5'-CGTCTTTCTG-3') and 5'-GT15G-3' as a 3' anchor primer, a wild-type p53-induced fragment was detected as a prominent band having around 240 bp in length, designated B4 fragment in a 12% PAGE (Fig. 1). This band was readily detected in a sample prepared at 6 h after the temperature shift-down and its level was similar at 12 h, suggesting that the B4 fragment sequence represents an RNA species induced by the wild-type *p53*. Consequently, the induced B4 fragment was cloned into a T/A cloning vector and the insert was

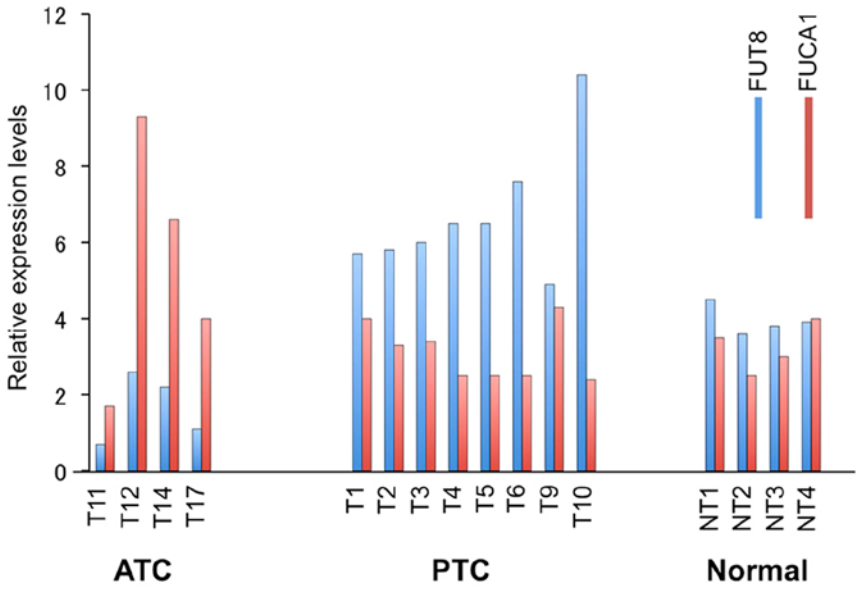

Figure 4. Microarray analyses of *FUT8* (blue) and *FUCA1* (red) expression. Four ATC samples (T11, T12, T14, T17), 8 PTCs samples (T1, T2, T3, T4, T5, T6, T9, T10), and 4 normal thyroid tissues (NT1, NT2, NT3, NT4) are shown.

sequenced. The sequence obtained was blasted to the NCBI data base (URL: www.ncbi.nih.gov) (Fig. 2). As expected, the 5' end of the determined B4 fragment contained the 3' side 9-base sequence (5'-GTCTTTCTG-3') out of the upstream 5' arbitrary 10 base primer sequence (5'-CGTCTTTCTG-3') except for the 5' most C residue.

In addition, the B4 fragment contained the poly(A) addition signal near the 3' end. Further, the obtained sequence was found to match exactly with the 3' end region within the non-coding sequence of the exon 8 of human *α-L-fucosidase-I (FUCA1)* mRNA (gene bank: KR710572). To confirm that *FUCA1* mRNA was transcriptionally induced in cells by the wild-type activity of *ts-p53A138V*, we performed Northern hybridization using ^{32}P-labeled B4 fragment as a probe for RNAs prepared from S/ts-p53 cells that were incubated at 32.5˚C. Fig. 3A shows that *FUCA1* mRNA species having approximately 20S and 18S sizes, were induced in S/ts-p53 cells 4 h after incubation at 32.5˚C. On the contrary, low levels of 20S RNA species and no detectable 18S RNA were synthesized in S/neo cells prepared either before or after the temperature shift to 32.5˚C, thus suggesting that both RNA species were p53-inducible in S/ts-p53 cells. In agreement with the results of upregulation of the *FUCA1* mRNA, Fig. 3B shows that also a 55 kD FUCA1 protein band was detected in lysates of S/ts-p53 cells prepared after incubation at the permissive temperature (32.5˚C) but was hardly detected at significant levels in the same cells incubated at the non-permissive temperature (37.5˚C).

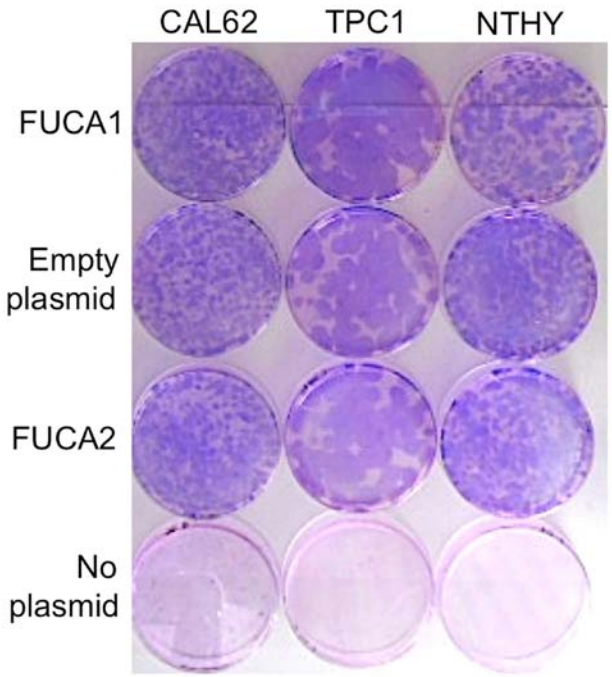

Figure 5. Clonogenic potential of normal NTHY, TPC1 and CAL62 cell lines after transfection with a quarter pmol each of FUCA1 DNA, FUCA2 DNA, or the empty plasmid DNAs, all carrying neomycin resistant gene, or H_2O as a negative control. Colonies of transfected cells appeared after G418 selection after 4 weeks were stained with Giemsa.

FUCA1 expression levels are high in papillary thyroid cancers but low in anaplastic thyroid cancers. As most PTCs carry the wild-type *p53*, while most ATCs carry mutant *p53* (13), we analyzed *FUCA1* RNA expression levels by microarray analysis of papillary and anaplastic thyroid cancers versus normal thyroid tissues. Average levels of *FUCA1* mRNA were low in ATCs while expression levels in PTC samples were comparable or slightly higher than those present in normal thyroid tissues (Fig. 4). Even though we have not studied the p53 status of the samples analyzed, it is reasonable to assume that the *FUCA1* expression levels may be related to the *p53* status in the two different thyroid cancer histotypes.

Overexpression of FUCA1 does not affect the clonogenic potential of thyroid cancer and normal cells. Since we found that *FUCA1* was expressed at low levels in ATCs, compared with PTCs and normal thyroids we have used a set of normal,

ATC- and PTC-derived cancer cell lines to investigate whether the enforced expression of *α-L-fucosidase* genes in these cell lines could have an inhibitory growth effect on any of the cell lines tested. To this aim, we analyzed the colony forming efficiencies of an ATC-derived cell line (CAL62), of a PTC-derived cell line (TPC1) and a normal thyroid cell line (NTHY) after transfection with *FUCA1* or *FUCA2* containing plasmids or with the empty plasmid vector. As shown in Fig. 5, there were no significant differences in colony forming efficiencies between *α-L-fucosidases*-transfected, and empty vector-transfected cells for normal NTHY, papillary TPC1, and anaplastic CAL62 tumor cell lines. This result suggests that the enforced expression of *α-L-fucosidases* did not significantly affect the clonogenic potential of the thyroid cancer or normal cells.

FUT8 RNA expression levels are inversely related to those of FUCA1 RNA. Since fucose levels of cell surface glycoproteins is enhanced by fucosyltransferases (FUTs), we also analyzed *FUT8* mRNA levels in the same thyroid cancer and normal biopsies samples by microarray analysis. As shown in Fig. 4, *Fut8* expression was higher in ATC but lower in PTC samples, thus showing the existence of an inverse relationship between the expression levels of *FUCA1* and *FUT8* RNA in the two different thyroid cancer histotypes analyzed. We have also analyzed the expression levels of *FUT1, FUT3, FUT4*, and *FUT6*, however, we did not detect such drastic changes as observed for *FUCA1* and *FUT8* (data not shown).

Discussion

FUCA1 is transcriptionally induced by p53. In the present study we showed that *FUCA1* is transcriptionally activated by the wild-type activity of *p53*, as evidenced by the result that 18S and 20S FUCA1 RNA species were induced in the Saos-2/ts-p53A138V cells. Accordingly, the induced FUCA1 protein was detected in the Saos-2 cells at the permissive temperature and was hardly detected at significant levels at the non-permissive temperature. Further, we analyzed microarrays of samples obtained from ATC- or PTC-bearing patients and from normal thyroid tissues. We found that the ATC specimens displayed low levels of *FUCA1* expression compared with either PTC or normal thyroid tissue specimens. Since most ATCs are reported to harbor a mutated *p53*, while PTCs generally harbor the wild-type *p53* (13), it is reasonable to conclude that the levels of *FUCA1* expression are related to the *p53* status, i.e., in ATC samples low levels of *FUCA1* are related to the presence of a mutated *p53*, whereas PTC samples, which contain the wild-type p53, display relatively high levels of *FUCA1*, as the normal thyroids. In fact we confirmed these differences at protein level in cell lines derived from the two different cancer histotypes (25).

In addition to anaplastic thyroid cancer, *FUCA1* expression is downregulated in aggressive forms of colorectal cancers (26), breast cancers (27), and neuroblastomas (28). It should be noted that a gradual decrease in *FUCA1* expression was observed with progression of disease from earlier to advanced stages in colorectal cancer (26). Also in the case of thyroid cancer, as shown previously (25) less aggressive thyroid PTCs expressed higher levels of FUCA1, compared with the more aggressive ATCs, which displayed lower levels of FUCA1 expression. It is interesting to note that it has been reported that ATCs derive from progression of more differentiated PTCs and of follicular thyroid cancers (FTCs) (29). It is therefore speculated that one of the steps in progression of thyroid cancer may be related to the loss of FUCA1 expression which is consequent to the loss of the wild-type p53 status. In both cases (thyroid and colon cancers) therefore, *p53* mutations are likely to be associated with the more aggressive phenotype and with progression (29,30).

As described above, it was suggested that low levels of *FUCA1* are related to the aggressiveness of the cancer type, rather than to the tumor cell growth *per se*. Using a different cancer cell system, Ezawa *et al* (16) recently reported that transfection of *FUCA1* induced apoptosis. It is likely that this is not the case with the thyroid cell system used by us since the results obtained by us and reported here regarding the clonogenic potential of *FUCA1* transfected TPC1 and CAL-62 cells suggested that enforced *FUCA1* expression did not interfere with the clonogenic potential of normal thyroid and PTC- and ATC-derived cancer cell lines, nor, as reported previously (25), enforced expression of *FUCA1* interfered with the cell growth potential of another ATC-derived cell line, 8505c. Our finding is thus consistent with that reported by Baudot *et al* (17), who showed that the wild-type *FUCA1* did not interfere with the clonogenic potential of Saos-2 cell growth, nor affected directly apoptosis. Besides, *FUCA1* interfered with the capacity of the transfected anaplastic cell line to grow in agar or penetration through Matrigel and to adhere to various matrices (25). Taken together with the result that higher levels of *FUCA1* were expressed in PTCs, it is conceivable, thus, that removal of fucose residues by *FUCA1* from cell surface molecules, might decrease the binding of cancer cells to endothelium or to extracellular matrix molecules, which is considered as a necessary step leading to the metastatic process of breast cancer cells (7,8) and thyroid cancer cells (25).

Roles of upregulation of FUT8 in carcinogenesis. We found also that fucosyltransferase 8 (FUT8), but not FUT1, FUT3, FUT4, and FUT6 were aberrantly upregulated in aggressive ATCs and downregulated in less aggressive PTCs. Upregulation of FUT8 has been reported for several other types of cancer: hepatocellular carcinoma, lung cancer, breast and prostate cancers (31). In addition, we found an inverse relationship in the relative expression levels of *FUCA1* and *FUT8*, in PTCs and ATCs. As described above, FUT8 is involved in core-fucosylation. Increased core-fucosylation of growth factor receptor (GFR) was reported to be associated with increased GFR-mediated signaling and with tumor cell growth and malignancy (2,31,32). Consequently, ATCs displaying lower levels of FUCA1 and higher levels of FUT8, may have gained a more aggressive phenotype, also as a consequence of the increased fucose levels present on their surface glycoproteins, while PTCs, displaying higher levels of *FUCA1* mRNA and lower levels of *FUT8* mRNA show a less aggressive phenotype, also as a consequence of the decreased fucose levels present on their surface glycoproteins. Further experiments are needed in the direction of ascertaining which are the important target molecules present on the cell surface of these two cancer histotypes.

Acknowledgements

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