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Glycosylation and Glycoproteins in Thyroid Cancer: A Potential Role for Diagnostics

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1. Introduction

Glycosylation is the most common and the most diverse form of co- and post-translational modifications. An analysis of the Swiss-PROT database has led to the estimation that more than 50% of all proteins are glycosylated. Genes coding proteins involved in all types of oligosaccharides biosynthesis represent 0.5 to 1% of the translated genome (Dennis et al., 1999). Glycoproteins are found ubiquitously in an organism either as soluble (intracellular or extracellular) or as membrane bound molecules. There is a great structural variety of glycoproteins based on the type, length and linkage of a carbohydrate components as well as the degree of saturation of potential glycosylation sites on the protein itself.

Carbohydrates can have a significant influence on the physicochemical properties of glycoproteins, affecting their folding, solubility, aggregation and degradation. Furthermore, glycan chains in glycoproteins play key roles in many biological processes such as embryonic development, immune response and cell-cell interactions in which sugar-sugar or sugar –protein specific recognition is involved (Wei & Li , 2009).

Altered glycosylation is an universal feature of cancer cells, and certain glycan structures and glycoproteins are well-known tumor markers. These include for example glycoproteins such as carcinoembryonic antigen (CEA), commonly used as a marker of colorectal cancer, prostate-specific antigen (PSA) and CA-125 used in the diagnosis of ovarian cancer (Drake et al., 2010). High expression of some glycosyl epitopes for example sialyl Lewis a, sialyl Lewis x, Lewis y, promotes invasion and metastasis. Antibodies against Lewis antigens are used for evaluation of specimens from breast, bladder, colorectal, esophageal and lung carcinomas (Drake et al., 2010). Glycans can regulate different aspects of tumor progression, including proliferation, invasion and metastasis. Cancer-related changes in glycosylation can reflect disease specific alterations in glycan biosynthetic pathways. These include variations in the expression and activity of glycosyltransferases, enzymes that add monosaccharides to acceptors, i.e. proteins or growing carbohydrate chains and glycosidases which catalyze the hydrolysis of the glycosidic linkage to release sugars.

There are three most common categories of protein glycosylation 1) *N*-glycosylation, where glycans are attached to asparagine residues in a consensus sequence N-X-S/T *via* *N*-acetylglucosamine (GlcNAc) residue; 2) *O*-glycosylation, where glycans are attached to serine or threonine *via* *N*-acetylgalactosamine (GalNAc) residue (mucin type glycosylation);

3) *O*-GlcNAcylation, where single *N*-acetylglucosamine residues are attached by *O*-linkage to serine and threonine residues (Fig.1). *N*-oligosaccharides have a common core structure of five sugars and differ in their outer branches. They are divided into three main classes: high mannose, complex and hybrid. High mannose oligosaccharides have additional mannoses linked to the pentasaccharide core and forming the branches. Complex-type oligosaccharides contain characteristic GlcNAc and Gal residues and are often terminated with sialic acid residues. Complex type oligosaccharides can be bi-, tri- or tetraantennary.

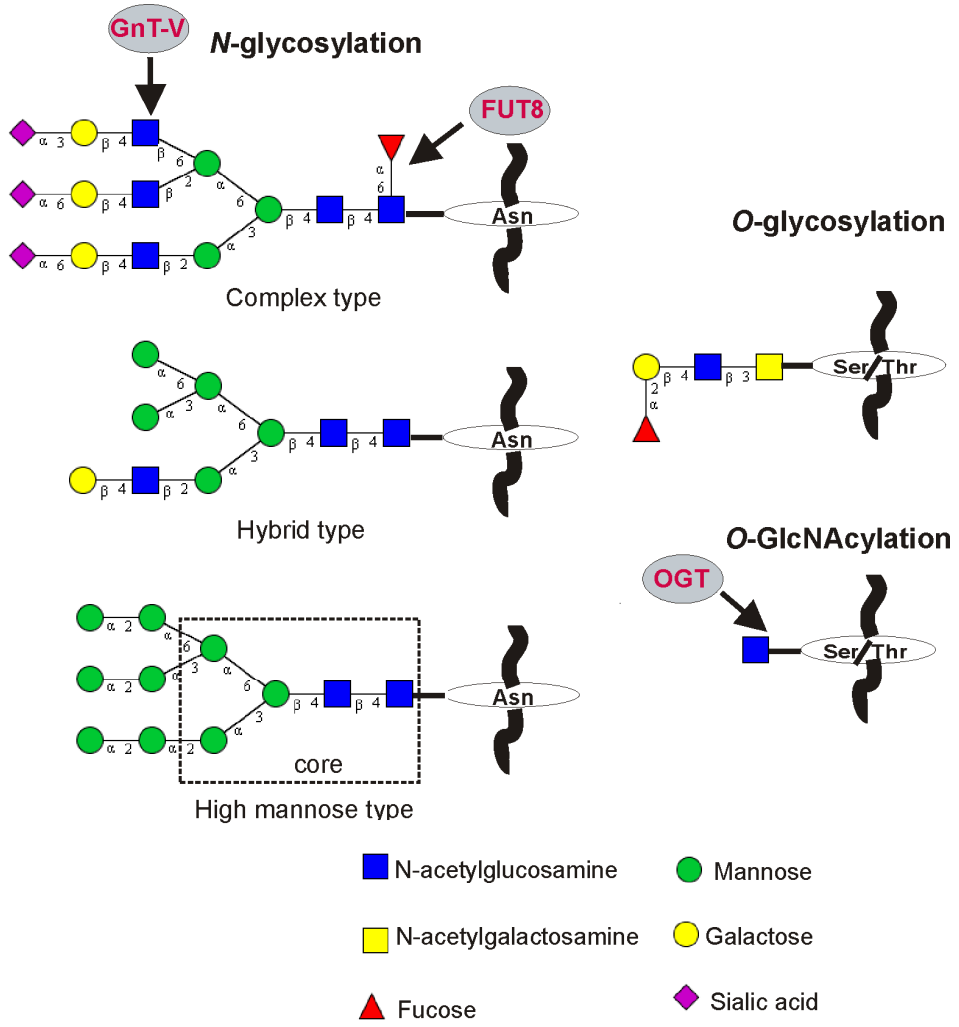

Fig. 1. Examples of carbohydrate structures. Arrows indicate the residues attached by glycosyltransferases the expression of which was studied in thyroid tumors:
GnT-V - *N*-acetylglucosaminyltransferase V , FUT8 – α 1,6 –fucosyltransferase,
OGT – *O*-GlcNAc transferase

Hybrid oligosaccharides contain one branch that has a complex structure and one or more high-mannose branches (Stanley et al., 2009). The mucin-type *O*-glycan, with *N*-acetylgalactosamine (GalNAc) at the reducing end, is a very common form of *O*-glycosylation in humans (Brockhausen, 1999; Wopereis et al., 2006). In *O*-glycosylated proteins, oligosaccharides range in size from 1 to >20 sugars, displaying considerable structural diversity. In total, 8 mucin-type core structures can be distinguished, depending on the second sugar and its sugar linkage, of which cores 1–6 and core 8 have been described in humans. The *N*-acetylgalactosamine may be extended with sugars such as galactose, *N*-acetylglucosamine, fucose or sialic acid but not mannose, glucose or xylose residues (Brockhausen et al., 2009). The structural variability of glycans is dictated by tissue-specific regulation of glycosyltransferase genes, acceptor and sugar nucleotide donors availability and by competition between enzymes for acceptor intermediates during glycan elongation. Glycosyltransferases catalyze the transfer of a monosaccharide from specific sugar nucleotide donors onto particular position of a monosaccharide in a growing glycan chain in one or two possible anomeric linkages (either α or β) (Dennis et al., 1999). *O*-GlcNAcylation is a specific type of glycosylation since *O*-GlcNAc residues are not elongated and do not form complex structure. This dynamic and inducible modification is more similar to phosphorylation than to classical glycosylation (Hart & Akimoto, 2009).

2. Glycosylation in thyroid cancer

It is known that glycosylation profiles change significantly during oncogenesis (Wei & Li, 2009). Detection of tumor specific alterations could be potentially useful for cancer diagnostics. Lectins are a very good tool for the detection of changes in glycosylation. They are a group of proteins or glycoproteins that have affinity to carbohydrates and can reversibly and specifically interact with certain glycan structural motifs. Some lectins are widely used in cancer diagnostics. For example *Helix pomatia* agglutinin (HPA) which detects αGalNAcβ1-4 Gal, is a part of a panel of markers for histological characterization of gastric cancer specimens. HPA as well as *Ulex europaeus* agglutinin (UEA)(recognize Fucα1-2Galβ) is also used in breast cancer biopsy assessment (Drake et al., 2010).

It has been suggested that lectins might be useful histochemical tools for detecting and analyzing poly-N-acetyllactosamines in thyroid normal and malignant tissues (Ito N et al., 1995,1996). Staining with lectins in combination with endo-beta-galactosidase digestion demonstrated that poly-N-acetyllactosaminyl structures ubiquitously and consistently produced in thyroid papillary carcinomas are highly heterogeneous in their chain length and branching status and quite different from those produced in other thyroid neoplasms (Ito N et al., 1995, 1996). Studies concerning glycosylation of intracellular proteins in benign and malignant thyroid neoplasms showed significant quantitative and qualitative differences in binding of *Erythrina cristagalli* (ECA) and *Ricinus communis* (RCA-120) agglutinins that recognize N-acetyllactosamine or galactose residues (Krześlak et al., 2003). In the majority of carcinoma samples lectin binding to cytosolic proteins was definitely weaker in comparison with adenomas and non-neoplastic specimens which suggests alterations in glycosylation of proteins in thyroid malignant tumors. Also binding patterns of *Sambucus nigra* (SNA) and *Maakia amurensis* (MAA) agglutinins which recognized sialic acids are different in malignant tumors compared with benign thyroid lesions (Krześlak et al., 2007).

Changes in glycosylation are not the random consequence of disordered biology in tumor cells. It is characteristic that of all the possible glycan biosynthetic changes, only a limited subset of changes are correlated with malignant transformation (Varki et al., 2009).

2.1 *N*-acetylglucosaminyltransferase V

The most widely occurring glycosylation change in cancers is enhanced β1,6 GlcNAc side chain branching (see Fig.1.). Increased amounts of β1,6 GlcNAc branched carbohydrates have been linked to tumor invasion and metastasis in case of several human cancers including breast and colon. This carbohydrate structure is a product of increased activity of *N*-acetylglucosaminyltransferase V (GnT-V) (Fernandes et al., 1991; Seelentag et al., 1998; Granovsky et al., 2000).

Ito Y et al. (2006) have studied by immunohistochemistry the expression of GnT-V in thyroid normal and cancer tissues. They found expression of this enzyme in 66 of 68 cases of papillary carcinomas, 10 of 23 - follicular carcinomas, 9 of 13 - follicular adenomas and 6 of 28 - anaplastic carcinomas but the enzyme was not expressed in normal thyroid tissue. It has been shown that matriptase, a tumor-associated type II transmembrane serine protease is a target protein for GnT-V and β 1,6 GlcNAc branching of oligosaccharide on matriptase increases resistance of this protein to proteolytic degradation. Increased expression of matriptase and GnT-V levels were characteristic for papillary carcinoma. Although matriptase is proposed to modulate the metastatic potential of some cancer cells, it is not likely that the matriptase and GnT-V promote invasion and metastasis of papillary thyroid cancers. The levels of expression of both matriptase and GnT-V were significantly high in microcarcinomas. In case of poorly differentaiated and undifferentiated (anaplastic) carcinomas which show local and distant metastasis, expression of these proteins significantly decreased. Thus matriptase and GnT-V probably play a role in early phases of papillary carcinomas growth, but not in their progression (Miyoshi et al., 2010).

2.2 Changes in sialic acid expression

Residues of sialic acid are usually found at the non-reducing terminal position of glycoconjugate sugar chains, α2,3- or α2,6-linked to a galactose (Gal), or α2,6-linked to a *N*-acetylgalactosamine (GalNAc) or *N*-acetylglucosamine (GlcNAc) residues (Harduin-Lepers et al., 2001). Changes in sialic acid expression may be important in cancer progression and metastasis for several reasons. First, sialic acid can prevent cell-cell interactions through charge repulsion effects; second, sialylated structures can be recognized by cell adhesion molecules belonging to selectin or siglec families; third, the addition of sialic acid may mask the underlying sugar structure, thus avoiding recognition by other lectins such as galectins (Dall'Olio & Chiricolo, 2001).

Nozawa et al. (1999) investigated immunohistochemically the reactivity of monoclonal antibody of FB21, which recognizes a sialic acid-dependent carbohydrate epitope with thyroid lesions in order to evaluated the potential diagnostic usefulness of sialic acid expression analysis. The increased FB21 antigen reactivity appeared to be characteristic for follicular and papillary thyroid tumors but not for medullary thyroid carcinoma. FB21 reacted with almost all cases of follicular carcinomas and less than half of cases of papillary carcinomas. A positive reaction was found on the cell surface membranes or apical parts of

neoplastic follicles. The normal thyroid follicles, goiters, medullary and anaplastic carcinomas were negative for FB21 reactivity. High frequency of reactivity of follicular carcinoma with FB21 suggested that it may be useful in diagnosis of this histological type of thyroid tumors.

Differences in sialic acid level on luminal surface between thyroid carcinomas and normal thyroid tissue or adenomas and goiters was observed in histochemistry studies using sialic acid binding lectins *Tritrichomonas mobilensis* lectin (TML), *Maackia amurensis* agglutinin (MAA) and *Sambucus nigra* agglutinin (SNA) (Babál et al., 2006). Malignant tumors especially papillary carcinoma had an increased level of sialic acid mainly alpha-2,3-linked and recognized by MAA. These results suggest that increased membrane sialic acid on thyroid gland cells may be an important diagnostic indicator, that could be useful in the distinction of malignant from benign thyroid lesions (Babál et al., 2006).

Cellular sialic acid level is mainly controlled metabolically by sialyltransferases and sialidases. Human sialyltransferases are a family of at least 18 different members that catalyse the transfer of sialic acid residues from their activated form CMP-sialic acid to terminal position of oligosaccharide chains (Harduin-Lepers et al., 2001). Changes in sialyltransferases expression have been observed in cancer tissues and the regulation of their expression is achieved mainly at the transcriptional level (Dall'Olio & Chiricolo, 2001). Sialidases catalyse the removal of sialic acid residues which is an initial step in the degradation of glycoproteins. There are only few studies focusing on sialic acid metabolism in thyroid tissue. Thyroid sialyltransferase 1 (β-galactoside α-2,6-sialyltransferase) and 4 (β-galactoside α-2,3-sialyltransferase) mRNA level and activity are increased in Graves disease (Kiljański et al., 2005). Sialyltransferase 1 showed also a very evident increase of expression in autonomously functioning thyroid nodules (AFTNs) *versus* their surrounding tissues (Eszlinger et al., 2004). Since sialyl Lewis epitopes are more frequently expressed in papillary carcinomas than Lewis a and Lewis b it is speculated that a specific glycosyltransferase, i.e. α2,3 sialyltransferase may be highly activated as compared with α1,4 fucosyltransferase. Moreover, this sialyltransferase seems to be more strongly enhanced in papillary carcinoma than in follicular carcinoma (Kamoshida et al., 2000).

2.3 Fucosylation

Fucosylated glycans are synthesized by fucosyltransferases. Thirteen fucosyltransferase genes have thus far been identified in the human genome. Based on the site of fucose addition fucosyltransferases are classified into α1,2 (FUT1, FUT2), α1,3/4 (FUT 3, 4, 5, 6, 7, 9), α1,6 (FUT8) and *O*-fucosyltransferase (POFUT1) groups (Becker & Lowe, 2003; Ma et al., 2006). Two additional α1,3 fucosyltransferase genes, *FUT10* and *FUT11* and one *O*-fucosyltransferase gene have been identified in the human genome but their protein products have not yet been demonstrated to be enzymatically active although they share primary sequence similarity with active fucosyltransferases. Certain types of fucosylated glycoproteins for example alfa-fetoprotein and several kinds of antibodies, which recognize fucosylated oligosaccharides such as sialyl-Lewis a/x, have been used as tumor markers (Miyoshi et al., 2008). The Lewis blood group antigens are a related set of glycans that carry α1,2/α1,4 fucose residues (Fig.2). Although growing evidence supports the functional significance of fucosylation at various pathophysiological steps of carcinogenesis and tumor progression, the significance of this modification in thyroid cancer has not been widely

explored. The results of studies of Vierbuchen et al. (1992) concerning blood group antigens in medullary thyroid carcinoma (MTC) and normal thyroid tissue supported the general concept demonstrated in other carcinomas, that fucosyl- and sialyltransferases might be preferentially activated. The Lewis antigens were absent in normal tissue and present in MTC. Miettinen and Kärkkäinen (1996) analysed immunohistochemically a wide range of thyroid tumors and non-neoplastic tissues by using CD15 antibody, which recognized Lewis x blood group antigen. Nodular goiter and papillary hyperplasia cases either showed no reactivity or were focally positive. Most papillary carcinomas were CD15 positive usually in the majority of tumor cells. However only 50% of follicular carcinomas were positive and anaplastic carcinomas were negative. Similar results were obtained by Fonseca et al. (1996, 1997) who found by using SH1 antibodies that most of papillary and follicular carcinomas were extensively immunoreactive for Lewis x antigen in contrast to the absence of expression of this antigen in normal thyroid.

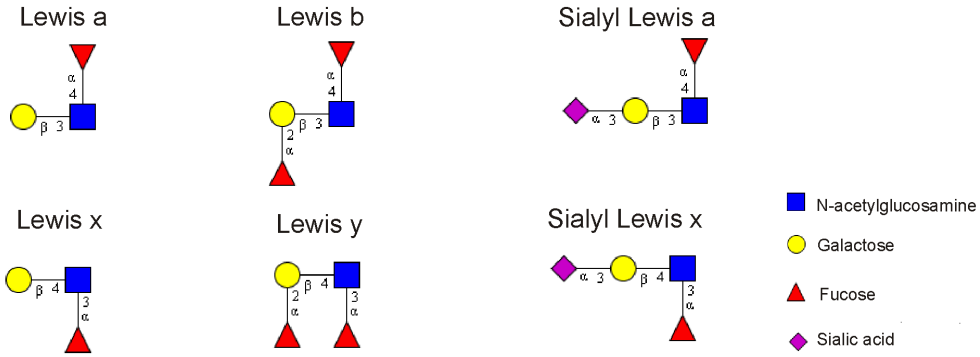

Fig. 2. Lewis antigens.

Ito Y et al. (2003) performed immunohistochemical studies of FUT8 expression in thyroid cancers. FUT8 catalyses the transfer of a fucose residue to the C6 position of the innermost GlcNAc residue of *N*-linked oligosaccharides on glycoproteins to produce core fucosylation (Fig.1.). The expression of FUT8 was very low in normal follicules. A high expression of FUT8 was observed in 33.3% of papillary carcinomas and the incidence was directly linked to tumor size and lymph node metastasis. But the number of cases of follicular carcinomas with high expression of FUT8 was rather low. These results suggest that FUT8 expression may be a key factor in the progression of thyroid papillary carcinomas, but not follicular carcinomas (Ito Y et al., 2003; Miyoshi et al., 2010).

2.4 *O*-GlcNAcylation

O-GlcNAcylation is a post-translational protein modification consisting of *N*-acetylglucosamine moiety attached by *O*-glycosidic linkage to serine and threonine residues (Hart et al., 2007). *O*-GlcNAc is a unique type of glycosylation since it is not elongated to more complex glycan structures and is nearly exclusively on cytoplasmic and nuclear proteins. Furthermore, the addition and removal of *O*-GlcNAc moieties cycles in a very dynamic and inducible manner in response to different stimuli such as hormones, growth factors, and mitogens. That makes *O*-GlcNAcylation more similar to

phosphorylation than to classical glycosylation. *O*-GlcNAcylation is one of the most common post-translational modifications. So far, nearly one thousand of cellular proteins have been identified to be *O*-GlcNAcylated (Butkinaree et al., 2010). These proteins belong to diverse functional groups and include nuclear pore proteins, transcription factors, RNA binding proteins, cytoskeletal proteins, chaperones, phosphatases, kinases and other enzymes (Zachara & Hart, 2006). There is growing evidence that *O*-GlcNAc modification is involved in a wide range of biological processes, such as signal transduction, transcription, cell cycle progression and metabolism (Hart et al., 2007). *O*-GlcNAc can modulate protein function by regulating protein activity, protein-protein interaction, localization and protein degradation (Zeidan & Hart, 2010).

There is a relationship between *O*-GlcNAcylation and phosphorylation. All *O*-GlcNAc modified proteins are also phosphoproteins and sometimes *O*-GlcNAc and *O*-phosphate moieties can compete for a binding site or alternatively *O*-GlcNAcylation and phosphorylation can compete *via* steric hindrance when the substrate modification sites are within several aminoacids from each other (Hu et al., 2010). However, unlike phosphorylation, where many kinases and phosphatases regulate the addition and removal of phosphate, *O*-GlcNAcylation is regulated only by two enzymes. The addition of *O*-GlcNAc to proteins is catalyzed by *O*-GlcNAc transferase (OGT) and its removal is catalyzed by *O*-GlcNAc- selective *N*-acetyl-β-D-glucosaminidase (*O*-GlcNAcase, OGA) (Iyer & Hart, 2003). OGT is encoded by a single gene on X chromosome (Lubas & Hanover, 2000; Shafi et al., 2000). Although there is only one *OGT* gene, human OGT has at least three different isoforms because of alternative splicing (Love et al., 2003, Hanover et al., 2003). The main difference between isoforms is the number of tetratricopeptide repeats in N-terminal region. The OGT isoforms differ also in their tissue distribution and probably they may have different functions (Lazarus et al., 2006).

The cloned sequence of *O*-GlcNAcase was found to be identical to that of MGEA5 (meningioma-expressed antigen 5), which was identified genetically in human meningiomas (Heckel et al., 1998; Comtesse et al., 2001). *MGEA5* localizes to chromosome 10q24.1-q24.3 region. *O*-GlcNAcase seems to be a bifunctional enzyme. The N-terminus contains *O*-GlcNAc hydrolase activity and C-terminus bears a putative histone acetyltransferase (HAT) domain (Toleman et. al, 2004). *O*-GlcNAcase is reported both HAT and *O*-GlcNAcase activity *in vitro*.

There is growing evidence that perturbation in *O*-GlcNAc signaling is involved in the pathology of cancers. Overexpression of OGT causes defective cytokinesis increasing polyploidy of cells, a feature common to many cancer cells (Slawson et al., 2005). *O*-GlcNAc is present on many transcription and cell cycle regulatory proteins. The protooncogene c-Myc which regulates transcription of genes involved in cell proliferation, apoptosis and metabolism is modified by *O*-GlcNAc at Thr58 and this potentially stabilizes the protein (Kamemura et al., 2002). The important tumor suppressor p53, which is mutant or dysregulated in many cancers bears *O*-GlcNAc at Ser149. Increased *O*-GlcNAcylation of p53 at Ser149 results in decreased p53 ubiquitination and stabilizes the p53 protein (Yang WH et al., 2006).

Several malignancies have been shown to have increased level of *O*-GlcNAcylation compared to normal tissue (Gu Y et al., 2010; Mi et al., 2011; Caldwell et al., 2010). Elevated

OGT mRNA expression was found in breast cancer cell lines and breast invasive ductal carcinoma compared with normal breast cells and normal breast tissue (Caldwell et. al., 2010; Krześlak et al., 2011a). It has been shown that poorly differentiated breast tumors (grade II and III) had significantly higher OGT expression than grade I tumors and lymph node metastasis is significantly associated with decreased *MGEA5* mRNA expression (Krześlak et al., 2011a). The intracellular *O*-GlcNAcylation has been found to be also associated with the pathogenesis of chronic lymphatic leukemia –CLL (Shi et al., 2010).

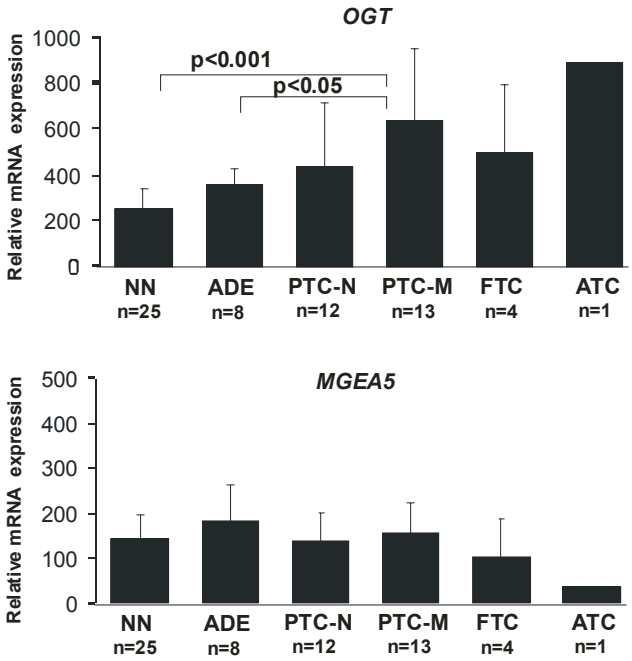

Fig. 3. *OGT* and *MGEA5* expression in thyroid lesions. The expression of *OGT* and *MGEA5* was studied by real time RT-PCR method in series of 25 samples of non-neoplastic lesions - NN (nodular goiters), 8 samples of follicular adenoma - ADE, 25 samples of papillary carcinoma – PTC (12 cases of non-metastatic –N and 13 cases of cancers with lymph node metastasis- M), 4 samples of follicular carcinomas - FTC and 1 sample of anaplastic cancer. TaqMan Gene Expression assays including fluorogenic, FAM labeled probes and sequence specific primers for *MGEA5, OGT* and *GAPDH* were purchased from Applied Biosystems. Assays numbers of OGT, MGEA5 and GAPDH were Hs00201970, Hs00269228 and Hs99999905, respectively. Abundance of *OGT* and *MGEA5* mRNA in samples were quantified by the ΔCt method. ΔCt (Ctgene – CtGAPDH) values were recalculated into relative copy number values (number of *OGT* or *MGEA5* mRNA copies per 1000 copies of GAPDH mRNA). The groups were compared using Mann-Whitney rank sum test. P value < 0.05 was considered statistically significant (non published data).

O-GlcNAcase activity was found to be increased in thyroid papillary, follicular and anplastic cancers in comparison with non-neoplastic lesions and benign tumors. *O*-GlcNAc-modified proteins in thyroid cells had a predominantly nuclear distribution and were more

abundant in non-neoplastic lesions than in tumors (Krześlak et al., 2010). However, our preliminary studies concerning *O*-GlcNAc cycling enzymes expression did not show any significant differences in mRNA expression of *MGEA5* between thyroid cancers and non-neoplastic lesions (Fig.3). Contrary, OGT mRNA expression seems to be elevated in cancer samples in comparison to nodular goiters or benign tumors. Moreover, expression of OGT mRNA was higher in papillary carcinoma with lymph node metastasis compared to non-metastatic carcinoma. High expression was also found in case of anaplastic carcinoma. These results might suggest that *O*-GlcNAc transferase is associated with thyroid cancer progression.

Caldwell et al. (2010) have provided evidence that the reduction abnormally elevated OGT and *O*-GlcNAc levels in breast cancer cells inhibits cancer cell growth *in vitro* and *in vivo* and also diminishes breast cancer invasion. Decreasing *O*-GlcNAc levels through knockdown of OGT in cancer cells promotes elevation of the cell-cycle regulator $p27^{kip1}$ and reduces expression of FoxM1 and its transcriptional target matrix metalloproteinase-2. Our studies concerning *O*-GlcNAc role in anaplastic thyroid cancer cells 8305C showed that down-regulation of *O*-GlcNAcase enhanced both basal and IGF-1 stimulated Akt1 activation and cell proliferation (Krześlak et al., 2011b). In cells treated with *O*-GlcNAcase inhibitor – PUGNAc or specific for *O*-GlcNAcase siRNAs phosphorylation of kinase GSK3β and cyclin D1 level were higher than in control cells. These findings suggest that increased proliferation of 8305C cells with down-regulation of *O*-GlcNAcase at least partially depends on IGF-1 – Akt1 – GSK3β – cyclin D_1 pathway.

Although abnormal *O*-GlcNAcylation seems to be a feature of cancer cells its role in tumorigenesis and cancer progression has not been fully elucidated and further investigations in this area are needed. However, it seems that *O*-GlcNAc transferase expression might be a marker for some tumor progression including thyroid. Moreover, regulation of *O*-GlcNAc may be a promissory novel therapeutic strategy for cancer and OGT may be in future a potential drug target.

3. Glycoproteins as thyroid cancer biomarkers

Glycoproteins seem to provide an abounding source for discovering biomarkers for thyroid lesions. Gene expression profiling studies identified some genes coding glycoproteins for example MET, SERPINA1, TIMP1, FN1, CD44, SDC4, DPP4 and PROS1 as important thyroid cancer markers (Griffith et al., 2006). These significantly deregulated genes in thyroid cancer cells may help to develop a panel of markers with sufficient sensitivity and specificity for the diagnostic purpose. Studies of cell surface and secreted protein profiles of human thyroid cancer cell lines (FTC-133, TPC-1, XTC-1, ARO, DRO-1) revealed distinct glycoprotein patterns for each cell line (Arcinas et al., 2009). Of the 333 glycoproteins identified in the five thyroid cancer lines, nearly one third, 105, were found exclusively in a single cell line. FTC-133 and ARO had the fewest (7) and the most (34) uniquely identified glycoproteins, respectively. It is suggested that several glycoproteins have a potential as biomarkers for a specific type of thyroid cancer or as general thyroid cancer biomarkers. For example NCAM-1 is a glycoprotein biomarker candidate for differentiated cancers, syndecan-1 and cadherin-13 for follicular carcinoma, elastin microfibril interfacer-1, hyaluronan and proteoglycan link protein 1, ephrin-B-1 for anaplastic carcinoma, CD157 for Hürthle cell thyroid carcinoma (Arcinas et al., 2009).

Usefulness of some glycoprotein expression analysis for thyroid cancer diagnostics have been already extensively tested.

3.1 Thyroglobulin

Thyroglobulin (Tg) is a large glycoprotein produced by thyroid normal follicular cells and differentiated malignant cells. The production of Tg is low in poorly differentiated cells and absent in anaplastic thyroid cancer (Francis & Schlumberger, 2008). Thyroglobulin provides a matrix for the synthesis of the thyroid hormones, thyroxine (T4) and triiodothyronine (T3), and acts as a storage vehicle for iodine, in the form of iodinated tyrosyl residues (Vali et al., 2000). Tg is a homodimer with molecular weight of 660 kDa and contains 10% of carbohydrate structures. Of the 20 putative N-linked glycosylation sites in the human thyroglobulin polypeptide chain, 16 were shown to be actually glycosylated in the mature protein (Yang SX et al., 1996). Eight of these confirmed glycosylation sites appear to be linked to complex-type oligosaccharide units containing fucose and galactose in addition to mannose and glucosamine. Five sites contain high mannose type units and two sites are linked to oligosaccharide units that may be either hybrid or complex structures (Yang SX et al., 1996). The oligosaccharides of Tg have been shown to affect the structure and function of Tg. They have an impact iodination and hormone synthesis, targeting of Tg to subcellular and extracellular compartments, interactions with a putative membrane receptor and immunoreactivity of Tg (Vali et al., 2000).

Owing to thyroglobulin tissue specificity, its serum level is widely used as a marker for recurrence of thyroid carcinoma following total thyroidectomy. The improved sensitivity of thyroglobulin assays and the adoption of an international calibration standard have made thyroglobulin measurement an essential part of thyroid cancer monitoring (Lin, 2008; Ringel & Ladenson 2004). However, it ought to be remembered that there are some limitations of serum thyroglobulin measurement. The presence of anti-thyroglobulin antibodies interferes with measurements of Tg protein made either by immunometric assay or by radioimmunoassay (Feldt-Rasmussen & Rasmussen, 1985; Mariotti et al, 1995; Spencer, 2004). Due to difficulties with antibody interference, there has been an interest in developing new techniques for thyroid carcinoma monitoring. One of these techniques is the detection of circulating thyroid cells by the measurement of thyroglobulin mRNA (Tg-mRNA) in peripheral blood (Ditkoff et al., 1996). However, there are contradicting results on the usefulness of Tg-mRNA detection in peripheral blood. Some authors suggested the usefulness of this method in the follow-up of thyroid cancer patients (Ringel et al., 1998, 1999; Biscolla et al, 2000; Savagner et al., 2002) but others questioned its reliability (Bojunga et al., 2000; Eszlinger et al., 2002; Span et al., 2003, Elisei et al., 2004). Thyroglobulin mRNA may be expressed in very low concentrations by other cells in the body, including peripheral lymphocytes, through a process known as illegitimate transcription (Verburg et al., 2004).

Since the serum Tg level is increased in the majority of patients with either benign or malignant thyroid nodules, its use is not recommended in the preoperative differentiation between thyroid lesions. However, it is suggested that the carbohydrate structure analysis of thyroglobulin might be useful for thyroid cancer diagnostics. During malignant transformation, epithelial cells modulate the glycosylation profile of their secretion proteins and these post-translational changes may represent specific markers which may be useful diagnostic or prognostic tools. The composition of carbohydrate chains on thyroglobulin

from thyroid carcinoma has been reported to differ from that in normal thyroid tissue (Maruyama et al., 1998; Shimizu et al., 2007a). Shimizu et al. (2007b) investigated *Lens culinaris* agglutinin (LCA)-reactive thyroglobulin ratio in serum to evaluate its usefulness for distinguishing between thyroid carcinoma and benign thyroid tumor. Their results were very promising. The *Lens culinaris* agglutinin-reactive thyroglobulin ratio was significantly lower in patients with thyroid carcinoma than in patients with benign thyroid tumor. Moreover, in cases of thyroid carcinoma with lymph node metastasis, *Lens culinaris* agglutinin-reactive Tg ratios were significantly decreased compared to patients with thyroid carcinoma without metastasis. Further studies confirm usefulness of the determination of LCA-reactive Tg ratios using an enzyme-linked immunosorbent assay for distinguishing between benign and malignant lesions (Kanai et al., 2009).

3.2 Fibronectin

Fibronectin (FN) is a high molecular mass adhesive glycoprotein present in the extracellular matrix that plays a significant role in cell adhesion, migration, growth, differentiation and the maintenance of normal cell morphology (Pankov & Yamada, 2002). Although fibronectin is encoded by a single gene it can exist in 20 different isoforms in humans as a results of alternative splicing and post-translational modifications. Fibronectin usually exists as a dimer composed of two ~250 kDa subunits linked by a pair of disulfide bonds near the C-termini. Subunits are composed primarily of three types of repeating module (I, II and III). Sets of modules make up domains for binding to variety of extracellular and cell surface molecules including collagen, glycosaminoglycans, fibrin, integrins and FN itself (Wierzbicka-Patynowski & Schwarzbauer, 2003). FN isoforms can be divided in two groups: soluble plasma FNs which are synthesized predominantly in the liver by hepatocytes and the less-soluble cellular FNs synthesized by other cell types. Heterogenity of fibronectin is also caused by post-translational modification. FN isoforms are glycoproteins that contain 4-9% carbohydrate, depending on the cell type. Glycosylation sites reside predominantly within type III repeats and the collagen-binding domain. The physiological role of the carbohydrates is not certain, but they appear to stabilize FN against hydrolysis and modulate its affinity to some substrates (Pankov & Yamada, 2002). There are seven potential *N*-glycosylation sites in human FN, and these *N*-glycans are largely responsible for the carbohydrate content of this molecule. The *N*-glycan of plasma FN is composed of complex-type biantennary oligosaccharides and is largely sialylated, whereas fibroblast-derived cellular FN contains fucose linked to the innermost *N*-acetylglucosamine (GlcNAc) and is less sialylated (Fukuda et al., 1982, Tajiri et al., 2005). Fetal and neoplastic cells predominantly express a uniquely glycosylated isoform called oncofetal fibronectin (OnfFN). This isoform is characterized by the presence of oncofetal domain situated in the COOH terminal region, which is absent in normal fibronectin. The oncofetal domain of FN is composed of an oligosaccharide linked to a hexapeptide by *O*-glycosylation (Matsuura et al., 1988).

Fibronectin 1 was first reported to be overexpressed in papillary carcinomas (Huang et al., 2001; Wasenius et al., 2003). Prasad et al. (2005) found that FN1 expression was significantly associated with malignancy and highly specific for carcinomas compared to adenomas and non-neoplastic tissues. Immunohistochemical analysis showed that FN1 was expressed in 61 of 67 papillary carcinoma cases, 3 of 6 follicular carcinoma, 4 of 4 anaplastic carcinoma and

6 of 8 Hürthle cells carcinoma cases. Contrary expression of FN1 was observed only in 2 of 29 samples of nodular goiters an 1 of 21 adenoma cases. Coexpression of FN1 with other markers such as galectin-3 or HBME1 was observed only in carcinomas (100% specific) while concurrent absence of FN1 and galectin-3 or HBME1 (Fn_Gal-3_ or FN_HBME1_ immunophenotype) was highly specific (96%) for adenomas (Prasad et al., 2005). Thus, this study suggest that fibronectin 1 is a very useful marker in the diagnosis of thyroid carcinomas. However, some other studies did not confirm high utility of fibronectin 1. Immunohistochemical studies of Nasr et al. (2006) showed that although FN1 was quite specific for papillary carcinomas, a large portion of PTC (31%) were negative. Even those cases that were positive showed weak and focal staining, and there was often significant background staining. The authors suggested that low sensitivity, focal staining and high background positivity significantly impair the utility of FN1 in diagnostics.

Using the reverse transcription-PCR technique, Takano et al. (1998, 1999) have suggested that oncofetal fibronectin (onfFN) may be an accurate molecular preoperative diagnostic marker for papillary thyroid carcinoma. They analyzed the expression of onfFN in fine-needle aspiration biopsies. The sensitivity and specificity of this method were 96% and 100%, respectively. They also showed high onfFN expression in anaplastic cancer tissues and cell lines (Takano et al., 2007). It has been suggested that oncofetal fibronectin may be a better tumor-specific marker in detecting minimal residual disease in differentiated thyroid carcinoma than thyroglobulin (Hesse et al., 2005). Thyroglobulin mRNA was thought to originate from circulating thyrocytes, but other studies demonstrated that physiological blood cells such as leukocytes are capable of ectopic Tg mRNA transcription (Verburg et al., 2004) In order to monitor patients with DTC for minimal residual disease by blood assays, Hesse et al. (2005) developed and optimized a specific, sensitive real-time RT–PCR assay using FRET technology to quantify absolute amounts of onfFN templates. High expression rate of onfFN transcripts in DTCs was demonstrated, while onfFN mRNA was not found to be illegitimately transcribed by peripheral blood cells. The authors suggested that onfFN mRNA analysis may be a specific tool for monitoring micrometastases in the context of minimal residual disease or for assessing tumor response to therapy. Continuing their studies they performed the analysis of mRNA level in peripheral blood of PTC patients who were previously treated by thyreoidectomy and treated by levothyroxine to determine if onfFN levels are correlated with the status of the disease or with thyroid-stimulating hormone (TSH) serum concentrations (Wehmeier et al 2010). The mean value of onfFN mRNA in bloods from healthy subjects was used as control. OnfFN transcripts were highly abundant in the peripheral blood of the patients, but the levels of onfFN mRNA did not differ significantly among the patients who were free of the disease, had local residual disease or metastatic disease. However, there was a trend towards higher expression rates of onfFN mRNA in patients with metastases than those free of disease. Discrimination between the disease states was better without TSH stimulation. The authors suggest that circulating onfFN mRNA, may be a useful tool to detect circulating thyroid cancer cells.

3.3 CD44

The CD44 glycoprotein is an acidic molecule whose charge is largely determined by sialic acid. CD44 plays a critical role in a variety of cellular behaviors, including adhesion, migration, invasion, and survival. The main ligand for CD44 is a hyaluronan but CD44 has

an afifinity to other extracellular matrix constituents such as osteopontin, collagens, and matrix metalloproteinases (MMPs) (Cichy & Puré, 2003). The gene for CD44 contains 20 exons, 12 of which are expressed by the most common form of CD44, referred to as standard CD44 (CD44s). Isoforms of CD44 are generated by the insertion of alternative exons (V1–V11) at a single site within the membrane-proximal portion of the extracellular domain (Ponta et al., 2003). Molecules containing the variable exons or their peptide products are designated CD44v. The most abundant standard protein consists of three regions, a 72 amino acid (aa) C-terminal cytoplasmic domain, a 21 aa transmembrane domain, and a 270 aa extracellular domain (Goodison et al., 1999). The degree of glycosylation can affect the ligand binding characteristics of the protein and therefore alter its function. The regulation of the amount and the type of post-translational modification can add further diversity to the range of potential functions of CD44 isoforms. Expression of multiple CD44 isoforms is frequently upregulated during neoplastic transformation. CD44, particularly its variants, may be useful as a diagnostic or prognostic marker of malignancy (Goodison et al., 1999).

Expression of CD44 both on mRNA and protein levels was intensively studied in thyroid pathological lesions in order to evaluate its utility in distinguishing benign and malignant tumors. Immunohistochemical and immunocytochemical analyses showed that CD44 molecule was preferentially expressed in papillary thyroid carcinoma compared with other thyroid neoplasms and non-neoplastic lesions (Figge et al., 1994; Böhm et al., 2000; Kim et al., 2002). Variant isoforms of CD44 (CD44v3 and CD44v6) were detected in follicular and papillary carcinomas as well as in follicular adenomas but not in non-neoplastic lesions (Gu et al., 1998). The frequency of both variants expression was significantly higher in PTC with node metastasis than in PTC without metastasis (Gu et al., 1998). Since CD44v6 is not expressed by normal thyrocytes and is variably detected in benign and malignant lesions Gasbarri et al. (1999) suggested that it could be a preoperative marker to identify malignant lesions. Its immunodetection in cytologic specimens obtained by fine-needle aspiration biopsy could be useful for selecting those nodular lesions of the thyroid gland that need to be surgically resected (Gasbarri et al., 1999). Böhm et al. (2000) found that reduced level of CD44s in differentiated thyroid cancer patients seemed to be an independent prognostic factor for unfavorable disease outcome. Age older than 60 years, distant metastases and advanced pTNM stage were related to the loss of CD44s expression.

The main problem in preoperative diagnostics of thyroid neoplastic lesions is distinguishing between follicular carcinomas and follicular adenomas. Nasir et al. (2004) tested usefulness of CD44v6 among other markers in differentiating benign and malignant follicular neoplasms. They found membranous CD44v6 staining in 81% of FTC but in only 20% of follicular adenomas and suggested that its immunohistochemical detection may be useful for follicular neoplasm diagnostics. Maruta et al. (2004) analysed CD44v6 immunostaining in fine-needle aspiration cytology of 35 follicular carcinoma specimens and 44 cases of follicular adenomas and observed positive reaction in 74% of carcinoma cases and 30% of adenomas. There was no correlation between the expression of CD44v6 in follicular carcinomas and characteristics such as capsular invasion, vascular invasion, metastasis, or tumor size (Maruta et al., 2004).

Since CD44 is often detected in benign lesions its diagnostic importance is questioned by some authors (Nikiel et al., 2006). Matesa et al. (2007) analyzed CD44v6 mRNA expression in order to answer whether the presence of macrophages and Hürthle cells are responsible

for positive expression detected in benign lesions. They found a statistically significant relationship between presence of Hürthle cells and positive expression of CD44v6 in nodular goiter cytological samples.

3.4 Osteopontin

Osteopontin (OPN) is an acidic hydrophilic glycophosphoprotein rich in aspartic acid, glutamic acid and serine and contains ~30 monosaccharides, including 10 residues of sialic acid. One carbohydrate chain is attached by *N*-glycosyl bond to protein and 5-6 chains are linked by *O*-glycosyl bond (Rangaswami et al., 2006). OPN is a member of a small integrin binding ligand *N*-linked glycoprotein (SIBLING) family of proteins which include bone sialoprotein (BSP), dentin matrix protein 1 (DMP1), dentin sialoprotein (DSPP), and matrix extracellular phoshoglycoprotein (MEPE) (Wai & Kuo, 2008). OPN functions by mediating cell-matrix interactions and cellular signaling through binding with integrin and CD44 receptors. Many studies have indicated that OPN is highly expressed in several malignancies. OPN expression is associated with tumor invasion, progression or metastasis in breast, stomach, lung, prostate, liver and colon cancers (for a review see Wai & Kuo, 2008; Rangaswami et al., 2006).

Castellone et al. (2004) showed that osteopontin was a major RET/PTC - induced transcriptional target in PC Cl3 thyroid follicular cells. RET/PTC also induced a strong overexpression of CD44 which is a cell surface signaling receptor for OPN. These results prompt further studies to ascertain whether OPN could be a useful PTC tumor marker. OPN expression was studied by immunohistochemistry in 117 samples of thyroid papillary cancer, benign lesions and normal tissues (Guarino et al., 2005). OPN was found to be overexpressed in PTCs compared with normal thyroid tissue, follicular adenomas and multinodular goiters. Moreover, OPN up-regulation was correlated with aggressive clinicopathological features of PTC. The prevalence and intensity of OPN staining were significantly correlated with the presence of lymph node metastases and tumor size. However, follicular variant of PTC had lower prevalance of OPN expression compared to classical type. Studies of Briese et al. (2010) on a large number of thyroid samples showed that normal thyroid was negative for OPN, thyroid adenomas were weakly OPN positive, whereas many carcinomas were strongly positive. However, there was no association between OPN expression and tumor size and metastasis status.

mRNA level of *SPP1* (osteopontin gene) was validated in a panel of 57 thyroid tumors using quantitative PCR (qPCR) (Oler et al., 2008). SPP1 was overexpressed in PTCs but the difference was not considered significant. However, *SPP1* expression was associated with the presence of lymph node metastasis for tumors >1 cm. The expression levels of *SPP1* in follicular variant of PTC and follicular carcinoma were lower than in classical type of PTC.

Recently Wang et al. (2010) found that osteopontin expression is positively correlated in thyroid papillary carcinoma with phosphorylated c-Jun kinase (p-JNK). Activation of the JNK signaling pathway appears to be an important event in thyroid tumorigenesis and, perhaps, in tumor progression making p-JNK a possible target in cancer treatment. Coordinated expression of p-JNK and OPN immunoreaction suggest an involvement of both genes in the same molecular pathway in thyroid lesions (Wang et al., 2010).

3.5 NCAM

Neuronal cell adhesion molecule (NCAM, CD56) mediates homotypic and heterotypic cell-cell adhesion (Crossin & Krushel, 2000). NCAM structurally belongs to the immunoglobulin superfamily. The extracellular part of NCAM consists of five Ig-like domains and two fibronectin type III-like domains. Alternative mRNA splicing results in three major isoforms: a 120 kDa isoform which is predominantly expressed in normal and well differentiated tissues and a 140 and 180 kDa isoforms that are found predominantly in less differentiated or malignant cell types (Jensen & Berthold, 2007). NCAM is unique among adhesion molecules because it carries a large amount of the negatively charged sugar, polysialic acid (PSA) (Crossin & Krushel, 2000). The presence of PSA can affect the strength or stability of adhesion systems in which NCAM is involved.

NCAM is present on follicular epithelial cells of the normal thyroid but its expression is reduced by malignant transformation and may affect the migratory capability of tumor cells (Zeromski et al., 1998, 1999; Satoh et al., 2001). Scarpino et al. (2007) analyzed NCAM expression in tissue sections of 61 cases of papillary thyroid carcinoma (PTC) using immunohistochemistry and quantitative real-time PCR. Reduced NCAM protein expression was observed by immunohistochemistry in all histological variants of PTC and also in lymph node metastases. Reduced expression of NCAM protein was associated with a significant reduction of NCAM mRNA in the tumor tissue compared with the paired normal thyroid tissue. Other studies showed NCAM to be extremely useful in the distinction between PTC and follicular benign or malignant lesions (El Demellawy et al., 2008, 2009; Ozolins et al., 2010). El Demellawy et al. (2009) evaluated the diagnostic value of protein expression using antibodies against NCAM in normal follicular thyroid epithelium, benign thyroid lesions, and thyroid carcinomas. Their aim was to study the applicability of difference in NCAM expression as a marker that distinguishes PTC, including the follicular variant from other follicular thyroid lesions and follicular thyroid carcinoma. They found that NCAM is of value in distinguishing PTC from other thyroid follicular pathology with a sensitivity of 100% and a specificity of 100%.

The potential role of NCAM in tumor cell biology was investigated by silencing the *NCAM* gene in the TPC1 thyroid papillary carcinoma cell line (Scarpino et al., 2007). The results confirm that modifications of NCAM expression cause profound alterations in the adhesive and migratory properties. However, contrary to the observation that loss of NCAM is usually associated with increased tumor invasiveness *in vivo,* NCAM-silenced TPC-1 cells were more adhesive to different extracellular matrix components, and were less efficient in cell migration and invasiveness. This discrepancy requires further investigations.

3.6 TIMPs

Tissue inhibitors of metalloproteinases (TIMPs) have a dual role in the process of tumor progression with both pro- and anti-tumorigenic activities. Although originally characterized by their ability to inhibit matrix metalloproteinases (MMPs) activity, TIMPs have additional biological activities and have been shown to regulate a number of cellular processes including cell growth, migration, and apoptosis (Stetler-Stevenson, 2008). Four TIMPs have been currently characterized in human and designated as TIMP-1, -2, -3 and -4. They are expressed by a variety of cell types and are present in most tissues and body fluids.

TIMP-1 gene differs from the other members of the family in having a short exon 1 that is transcribed but not translated. The function of exon 1 appears to be related to the control of the specificity of tissue expression and may contain tissue-specific repressor elements. TIMP-1 is a glycosylated protein and the *N*-linked oligosaccharides are composed of sialic acid, mannose, galactose and *N*-acetylglucosamine. The TIMP-1 glycosylation seems to play a role in various functions including correct folding of the nascent protein, transport of the molecule to cell surface and enhanced stability of the protein (Lambert et al., 2004). TIMP-3 has also one glycosylation site.

TIMP-1 mRNA or protein overexpression was found in thyroid papillary cancers compared to normal tissue or goiters (Hawthorn et al., 2004; Maeta et al., 2001). Shi et al. (1999) investigated TIMP-1 gene expression in 39 primary thyroid tumors to see whether there is a correlation between TIMP-1 expression and the aggressiveness of the disease. They also transfected human TIMP-1 cDNA into a papillary thyroid carcinoma cell line (NPA) to study the effect of TIMP-1 expression on its invasive potential using an *in vitro* tumor invasion assay. The results showed that TIMP-1 mRNA level correlated directly with tumor aggressiveness: the highest number of TIMP-1 transcripts was found in stages III and IV *versus* benign goitres. However, overexpression of TIMP-1 by gene transfer resulted in a significant suppression of the malignant phenotype of NPA cells which suggests that TIMP-1 may function as a thyroid tumor invasion/metastasis suppressor. To explain these discrepancies, the authors suggested that the increased levels of TIMP-1 transcripts observed in cancer samples came from stroma cells to counteract tumor invasion and metastasis.

Kebebew et al. (2006) performed a real-time quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) assay of several candidate diagnostic markers to distinguish benign from malignant thyroid neoplasms, and to predict the extent of the disease. TIMP-1 mRNA expression level as well as ECM1 (extracellular matrix protein 1), TMPRSS4 (transmembrane protease, serine 4) and ANGPT2 (angiopoietin 2) mRNA levels were found to be independent diagnostic markers of malignant thyroid neoplasms. The AUC (the area under the receiver operating characteristic (ROC) curve) for 4 diagnostic genes in combination was 0.993 with sensitivity of 100%, specificity of 94.6%, positive predictive value of 96.5%, and negative predictive value of 100%. Thus the RT-PCR multigene assay involving TIMP1 is an excellent diagnostic marker for differentiated thyroid cancer and it will be a helpful adjunct to FNA biopsy of thyroid nodules.

The plasma concentration of MMP-1, 2, 3, 8 and 9 as well TIMP-1 and 2 was evaluated by enzyme-linked immunosorbent assay (ELISA) in patients with thyroid cancers (papillary, anaplastic and medullary) and in healthy subjects. The author suggests that, predominance of MMP-2 over TIMP-2 and TIMP-1 over MMP-1 is a common feature in patients with thyroid cancer (Komorowski et al., 2002).

Recently Nam et al. (2011) have studied the expression of matrix metalloproteinase-13 (MMP-13) and tissue inhibitor of metalloproteinase-13 (TIMP-3) in thyroid cancer by RT-PCR. They found that MMP-13 and TIMP-3 expression levels were significantly decreased in PTC samples compared with normal thyroid tissues.

3.7 Dipeptidyl peptidase IV

Dipeptidyl peptidase IV (DPPIV), assigned to the CD26 cluster, is a multifunctional protein expressed on epithelial cells and lymphocytes. DPPIV is a type II transmembrane

glycoprotein which consists of a large extracellular domain (C-terminus) connected by a flexible stalk region to a hydrophobic transmembrane domain and short intarcellular tail (N-terminus) (Kotacková et al., 2009). It preferentially cleaves N-terminal dipeptides from polypeptides with proline or alanine in the penultimate position and regulates the activities of a number of hormones, neuropeptides, cytokines and chemokines (Havre et al., 2008; Kotacková et al., 2009). DPPIV palys an important role in immune regulation, signal transduction, chemotaxis, cell adhesion and apoptosis. It is suggested that DPPIV is also involved in the neoplastic transformation and tumor progression (Pro & Dang, 2004; Kotacková et al., 2009).

Numerous early studies showed that DPPIV is abnormally expressed in thyroid carcinomas and may be useful for the diagnosis of thyroid tumors (Aratake et al., 1991; Kotani et al., 1992; Tanaka et al., 1995; Umeki et al., 1996; Tang et al., 1996; Hirai et al., 1999). DPPIV-like enzymatic activity and DPPIV protein expression were up-regulated in thyroid cancers. However, DPPIV positivity is limited to the group of well-differentiated carcinomas, particularly papillary carcinoma (Kholová et al., 2003; de Micco et al., 2008). DPPIV sensitivity to malignant follicular tumors including the follicular variant of PTC was low with a misdiagnosis rate of 20–30%, especially with tumors presenting Hürthle or tall-cell features. Although, the use of DPPIV can increase specificity and positive predictive value of the cytological diagnosis, the value of the marker in the individual cases is very limited (Kholová et al., 2003). The analysis of DPPIV mRNA level in 102 PTCs and 77 normal thyroid fragments with the use of Q-PCR reaction confirmed the increase of DPPIV expression in papillary thyroid carcinoma. However, the ROC analysis revealed that the diagnostic efficiency of DPPIV estimation is limited. Thus diagnostic usefulness of DPPIV as a single PTC marker is also doubtful (Ożóg et al. 2006).

3.8 E-cadherin and dysadherin

Cadherins are members of a large family of transmembrane glycoproteins, many of which participate in Ca^{2+}-dependent homophilic cell-cell adhesion and play an important role in the formation of tissue architecture (Shapiro & Weis, 2009). E-cadherin is necessary for normal epithelial function. Dysadherin is a cancer-associated cell membrane glycoprotein. It inactivates E-cadherin function in a post-transcriptional manner, has an anti-cell-cell adhesion function and plays an important role in tumor progression and metastasis (Ino et al., 2002).

Several authors have investigated the expression of E-cadherin in thyroid cancers (Scheumman et al., 1995; Serini et al., 1996; von Wasielewski et al., 1997; Kapran et al., 2002; Rocha et al., 2003; Kato et al., 2002; Choi et al. 2005; Brecelj et al., 2005). In general, it appears that E-cadherin expression is retained in follicular neoplasms but it is reduced in papillary carcinomas and lost in anaplastic and poorly differentiated carcinomas. Reduction or loss of E-cadherin has been correlated with widely invasive growth and lymph node metastases (Naito et al., 2001). E-cadherin reactivity is reported to be an important prognostic factor in papillary thyroid carcinomas (Scheumman et al., 1995; Rocha et al., 2003). Lack of E-cadherin expression has been considered as an adverse prognostic factor for survival.

Dysadherin expression in thyroid cancer was reported by two studies. Sato et al. (2003) analyzed by immunohistochemistry dysadherin and E-cadherin expression in 92 thyroid

carcinomas. Dysadherin was detected in 39 of 51 papillary carcinomas and in all 31 undifferentiated carcinomas but not in follicular carcinomas or normal thyroid tissue controls. Dysadherin expression correlated significantly with tumor size, regional lymph node metastasis, and distant metastasis of the primary carcinoma. A significant association was also observed between dysadherin expression and death from thyroid carcinoma. Dysadherin expression showed a significant negative correlation with E-cadherin expression (Sato et al., 2003).

Batistatou et al. (2008) compared the dysadherin expression in papillary carcinomas and papillary microcarcinomas (PMC) to find out whether there are any differences in the cell–cell adhesion system between these two malignancies that have different biological behavior. PMC has been defined as a papillary neoplasm measuring 1cm or less in diameter and usually remains clinically silent and is often identified incidentally in surgically removed thyroid glands for other reasons (e.g. nodular hyperplasia, thyroiditis). A statistically significant difference in dysadherin and E-cadherin expression between PC and PMC and a negative correlation between E-cadherin and dysadherin expression regardless of tumor size were noted (Batistatou et al., 2008).

3.9 Mucins

Mucins are defined as high molecular weight glycoproteins that contain tandem repeat structures extensively glycosylated through GalNAc *O*-linkages at the threonine and serine residues. To date, twenty one members of human mucin (MUC) family have been reported and they are designed chronologically in order of discovery. On the basis of their structural and physiologic characteristics, mucins have been divided into three sub-classes: the secreted/gel-forming mucins, the soluble mucins, and the membrane- associated mucins (Kufe, 2009; Ohashi et al., 2006).

Mucins are multifaceted glycoproteins that play a crucial role in maintaining homeostasis and promoting cell survival. They form a physical barrier, which separates an apical surface of epithelial cells from the external environment and protects against infection and proteolytic degradation. The membrane-associated mucins are anchored to the surface of the cell by a transmembrane domain and then have short cytoplasmic tail that interacts with cytoskeletal elements and cytosolic adaptor proteins. Therefore, they can act as putative receptors that engage diverse signaling pathway linked to differentiation, proliferation and apoptosis. Several reports indicate that aberrant upregulations of mucins especially transmembrane forms may promote the malignant phenotype of human carcinomas. In many types of tumor overexpression of transmembrane mucins contribute to the oncogenesis by disrupting epithelial polarity and cell-cell interactions, to constitutive activation of growth and survival pathways and to blocking stress-induced apoptosis and necrosis (Bafna et al., 2010; Kufe, 2009; Carraway et al., 2003).

The production of mucins has been relatively frequently observed in thyroid carcinomas. In a study of 142 cases of thyroid neoplasms, Mlynek et al. (1985) found mucinous substances, which occurred in about 50% of the papillary and medullary carcinomas, 35% of the follicular carcinomas and 21% of the anaplastic varieties. The most widely studied mucin, associated with thyroid malignancies is MUC1, encoded by gene located on the chromosome 1q21-24. Wreesmann et al. (2004) suggested that up-regulation of MUC1 expression in papillary thyroid

carcinoma correlates with 1q21 amplifications and aggressive behavior. The MUC1 gene overexpression was present in 97,5% of tall-cell variant of PTCs (TCV) as compared with only 35% of conventional PTCs. These results are in contrast to the previous analysis of Bièche et al. (1997) who detected no MUC1 gene amplification in 14 tumor samples (6 adenomas and 8 PTC) informative for the MUC1 gene. However, these studies showed higher MUC1 expression level in 6 out of 11 papillary carcinoma cases in comparison with 10 macrofollicular adenoma cases and normal thyroid tissue. Moreover, the authors observed that intracytoplasmic MUC1 staining occurred in 75% (3/4) of "high-risk" papillary thyroid carcinomas and in only 28,5% (2/7) of the "low-risk" PTC without extrathyroidal invasion or lymph node involvement, suggesting that MUC1 can be associated with more aggressive tumors. Indeed, comparing human thyroid cancer cell lines, Patel et al. (2005) found increased MUC1 expression in aggressive thyroid cancer cell lines (8305C, KAT4, ARO, BHP2-7, BHP10-3, BHP7-13, BHP18-21) but not in other papillary and follicular thyroid cancer cell lines (KAT5, KAT10, NPA, WRO, FRO). In addition, they demonstrated that targeting MUC1 with monoclonal antibody selectively affects cell viability and confirmed that MUC1 plays a crucial role in the thyroid behavior. Furthermore, a relationship was observed between cytoplasmic MUC1 and cyclin D1 immunostaining in the conventional PTCs and in papillary microcarcinomas (PMCs), implying that MUC1 may be involved in the regulation of Wnt pathway and has a role in B-catenin/cyclin D1 signaling. However, due to a very broad MUC1 expression in different histological variants, the authors were not able to determinate its prognostic utility (Abrosimov et al., 2007). Magro et al. (2003) found that MUC1 may co-exist in variety of glycosylated forms in different cellular compartments. They supposed that post-translational modification of mucins, rather than alterations in expression levels, may be stronger associated with tumor progression and specific glycosylation traits of MUC1 and could be an ancillary tool in histopathological diagnosis. Other investigators found MUC1 mRNA overexpression in 15 cases of PTC in contrast to 22-follicular adenomas and 22-normal thyroid tissues (Baek et al., 2007). More recently, Morari et al. (2010) basing on the study of 410 patients reported that MUC1 expression distinguished benign from malignant thyroid tissue with sensitivity of 89%; specificity of 52%; predictive positive value=75%; predictive negative value=74%. In conclusion, they suggested that MUC1 is not a reliable prognostic marker but may be useful in characterization of thyroid carcinomas, especially the follicular patterned thyroid lesions. The expression of other mucins in thyroid neoplasms has been poorly investigated. Nam et al. (2011) have suggested that MUC4 and MUC15 are the other important mucins associated with thyroid transformation. They showed significantly increased MUC4 and MUC15 mRNA level and positive immunoreactivities in PTC compared with normal tissue. High MUC4 expression correlated with small tumor size and papillary thyroid microcarcinoma subtype whereas overexpression of MUC15 was associated with age, the presence of multifocality and distant metastasis. The results of this study suggest, that MUC4 and MUC15 may play a crucial role in thyroid malignancy, especially the MUC15 may be a new prognostic marker and potential therapeutic target. However, there are some discrepancies between the previously published reports that indicated weakly positive or negative MUC4 staining of both benign and malignant thyroid carcinomas (Baek et al., 2007; Magro et al., 2003). The expression of MUC2, MUC3, MUC5AC, MUC5B and MUC6 in thyroid neoplasms has been tested, though none of these glycoproteins was associated with clinicopathological features of thyroid carcinomas (Magro et al., 2003; Alves et al., 1999). The clinical and prognostic significance of mucins in thyroid tumors is still an open question.

4. Galectins

Galectins are endogenous lectins defined by shared consensus amino acid sequences and an affinity for β-galactose containing oligosaccharides. Fifteen members of galectin family have been identified and classified into three subgroups based on their structure: prototype (galectin-1, 2, 5, 10, 11, 13, 14, 15), tandem repeat type (galectin-6, 8, 9, 12) and chimera type (galectin-3). The presence of at least one carbohydrate recognition domain (CRD) is a common characteristic of galectins (Krześlak & Lipińska, 2004; Elola et al., 2007; Boscher et al., 2011). Galectins have both intra- and extracellular localization. Galectins can be secreted by non-classical pathway and depending on the cell type or differentiation state, they are found in the cytoplasm and the nucleus, on the cell surface and in the extracellular matrix (Hughes, 2001; Haudek et al., 2010; Garner & Baum, 2008). Galectins have been shown to play roles in diverse biological processes, such as embryogenesis, adhesion and proliferation of cells, apoptosis, mRNA splicing and modulation of immune response (Krześlak & Lipińska, 2004; Elola et al., 2007; Boscher et al., 2011).

Among various galectins, galectin-1, 3 and 7 have gained attention as potential markers of thyroid malignancy. However, the data concerning galectin -1 and 7 expression in thyroid are very limited. Chiariotti et al. (1995) analyzed mRNA and protein levels of galectin-1 in 74 human thyroid specimens of neoplastic, hyperproliferative and normal tissues. Galectin-1 mRNA levels was increased in 28 of 40 papillary carcinomas and in 6 of 7 anaplastic carcinomas compared with normal or hyperplastic thyroid. Immunohistochemical analysis of normal thyroid and papillary carcinoma sections revealed a higher content of galectin-1 protein in neoplastic cells than in normal cells. Similarly Xu et al. (1995) showed that all studied by them thyroid malignancies of epithelial origin (i.e., papillary and follicular carcinomas) and metastatic lymph node from a papillary carcinoma expressed high levels of galectin-1. In contrast neither benign thyroid adenomas nor adjacent normal thyroid tissue expressed galectin-1. There are only two studies concerning galectin-7 expression in thyroid cancers (Rorive et al., 2002; Than et al., 2008). The profile of galectin-7 expression is different from that of galectin-1 and 3. The results of immunohistochemical analysis revealed that this galectin is markedly down-regulated in adenomas compared with multinodular goiters and carcinomas (Rorive et al., 2002). Than et al. (2008) did not show any diagnostic value of galectin-7 in thyroid malignancy, even for a simple differentiation between obvious benign and malignant thyroid lesions .

In contrast to galectin-1 and 7, galectin-3 is one of the best studied molecular markers for thyroid diagnosis (for rewiew see Sanabria et al., 2007; Chiu et al., 2010) . The expression of this protein was widely studied both in cancer tissues and in cytological specimens. Numerous studies have reported that galectin-3 is a very sensitive and reliable diagnostic marker for identification of thyroid carcinomas with high sensitivity and specificity (Orlandi et al., 1998; Gasbarri et al., 1999; Inohara et al., 1999; Bartolazzi et al., 2001; Saggiorato et al., 2001,2004; Pisani et al., 2004). However there are studies which did not confirm this diagnostic utility of galectin-3 (Niedziela et al., 2002, Mehrotra et al., 2004, Mills et al., 2005) The discrepancies observed in these reports could have resulted from different methods used for galectin-3 expression analysis. These methods differ in sample preparation details, the used antibody, dilution of antibodies, assessement of positive

results (percentage of marked cells and intensity of staining). Moreover, in the thyroid gland, oxyphilic cells are rich in endogenous biotin and galectin-3 immunocytochemistry may provide false-positive results in oxyphilic cell lesions due to biotin-based detection systems (Volante et al., 2004).

Recently, the diagnostic utility of galectin-3 in distinguishing benign from malignant follicular thyroid nodules was evaluated in a multicenter trial comprised a large cohort of patients (Bartolazzi et al., 2008). Galectin-3 expression analysis was applied preoperatively on 465 follicular thyroid FNAB samples and diagnostic accuracy was compared with final histology. Galectin-3 expression had an overall reported sensitivity of 78%, specificity of 93% and accuracy of 88% for distinguishing benign and malignant thyroid tumors. Similar results were obtained in two center trial reported by Franco et al. (2009). They compared galectin-3 staining results in suspicious or indeterminate FNABs samples with the histologic diagnosis of the thyroidectomy specimens. Galectin-3 expression was found to have sensitivity of 83%, specificity of 81%, positive predictive value of 84%, and negative predictive value of 80%.

Few studies have evaluated the correlation between galectin-3 expression and clinicopathological parameters of thyroid cancers such as tumor size and grade, capsular invasion or lymph node metastasis status. The galectin-3 expression level in follicular carcinoma was significantly increased with the degree of vascular or capsular invasion (Ito Y et al. 2005). High galectin-3 expression was also associated with lymph node metastasis in MTC (Faggiano et al., 2002; Cvejic et al., 2005). Contrary, Kawachi et al. (2000) reported significantly higher galectin-3 expression in the primary foci of the thyroid tumors with lymph node metastases, but they found lower levels of galectin-3 in their metasatsis offspring. Recently, Türköz et al. (2008) suggested that galectin-3 overexpression is more profound in early stages of papillary carcinoma, and its expression intensity decreases during tumor progression.

Two studies have analyzed the serum level of galecin-3 in thyroid cancer patients (Inohara et al. 2008; Saussez et al., 2008). They measured serum level of galectin-3 by ELISA method and compared with histological diagnosis after thyroidectomy. Although Sauessez et al. (2008) showed that higher serum level of galectin 3 was associated with thyroid disease there were no significant differences between benign and malignant thyroid tissues. Inohara et al. (2008) showed that serum galectin concentration in patients with papillary carcinomas did not differ significantly from that in patients with follicular carcinoma or adenoma and in healthy individuals.

Studies of Bartolazzi et al. (2008) and Franco et al. (2009) suggest that galectin-3 expression analysis may represent useful diagnostic method for those follicular nodules that remain indeterminate by cytological diagnosis and may improve the selection of patients for surgery. However before it can be used in clinical practice there is an urgent need for validation of methods of galectin-3 expression analysis. Some other studies suggest that diagnostic usefulness of galectin-3 may be significantly increased by combining with other potential thyroid cancer markers such as HBME1, cytokeratin-19, DPPIV, CD44v6, thyroid peroxidase (Aratake et al., 2002; Weber et al., 2004; Maruta et al. 2004; de Matos et al., 2005; Prasad et al., 2005; Papotti et al., 2005; Liu et al., 2008; Barut et al., 2010).

5. Conclusion

Fine needle aspiration biopsy (FNAB) is a practical and the most effective diagnostic technique for the preoperative detection of malignant nodule. However, the important limitation of FNAB is the lack of sensitivity in the evaluation of follicular neoplasms due to its inability to differentiate benign follicular lesions from their malignant counterparts. Moreover, for definite diagnosis of malignancy a complete excision with unequivocal evidence of capsular or vascular invasion is required. Therefore, new markers that may allow for accurate diagnosis of thyroid malignancy and eliminate potentially unnecessary surgery are needed. Glycosylation changes and the analyses of expression of some glycoproteins or galectins can provide a novel strategy for improvement of thyroid cancer diagnostics. So far, the most promising results have been obtained in the case of galectin-3 expression. Currently an improved galectin-3 immunodetection method represents a new methodological approach that can be used to optimize the preoperative selection of thyroid nodules. Further studies concerning alterations of glycosylation and glycoprotein patterns in thyroid pathological specimens may potentially lead to the discovery of novel biomarkers.

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7. References

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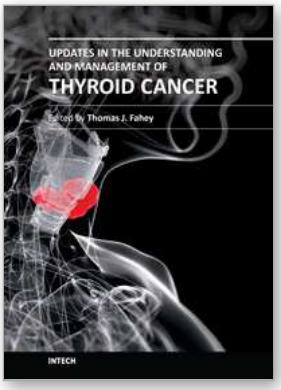

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