12 Circulating Mucins and Cytokeratins in Aggressive Thyroid Cancers

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12.1 Introduction

Follicular cell-derived thyroid carcinomas constitute a biological *continuum* progressing from the highly curable DTC to the much more aggressive, and almost always fatal, undifferentiated, or anaplastic thyroid carcinoma (ATC) [1]. Poorly differentiated thyroid carcinoma (PDTC) and aggressive variants of DTC, such as tall cell and columnar cell, frequently serve as intermediates in this progression model of dedifferentiation [2]. In fact, the gradual loss of typical papillary and follicular growth patterns and the simultaneous appearance of a solid growth pattern, with increased mitoses, necrosis, and nuclear pleomorphism, are frequently observed in aggressive thyroid carcinomas. Otherwise, residual foci of differentiated carcinoma may be frequently detected in aggressive tumor forms [3]. While radioactive iodine (I-131) is an effective treatment in thyroid cancers exhibiting a differentiated phenotype, there is a large body of information demonstrating that patients whose metastases concentrate ^{131}I have a higher survival rate and thus a better prognosis than patients with ^{131}I-refractory metastases [4]. Dedifferentiation of thyroid cancer may consist of loss of expression of the TSH receptor, natrium-iodide symporter (NIS), and loss of thyroglobulin (Tg) production and radioiodine cannot be longer employed for monitoring and treatment. In turn, this subset of tumors frequently shows avid ^{18}F-fluorodeoxyglucose (FDG) uptake in positron-emission tomography/computed tomography scans (FDG-PET/CT) [5]. These iodine-negative/FDG-positive tumors have a poor survival, and, consequently, alternative treatment options are required which may include observation, additional surgery, external beam radiation, interventional radiology (i.e., radio-frequency ablation), or systemic treatments [6].

Chemotherapy has shown limited success at best, while tyrosine kinase inhibitors (TKIs) have been introduced and tested in recent clinical trials. The DECISION trial using sorafenib showed a significant improvement in progression-free survival (PFS) of 10.8 months (vs. 5.8 months in the placebo group) [7]. In the SELECT trial, lenvatinib could demonstrate significantly increased PFS in patients with progressive radioiodine-refractory DTC. In comparison to sorafenib,

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lenvatinib even represented the most active agent with a better tumor response rate and an improved PFS of 18.3 months [8].

Based on these results, both drugs have been approved by the FDA for the treatment of locally recurrent or metastatic, progressive DTC that no longer responds to radioactive iodine treatment.

In order to assess effectiveness of TKI treatment, morphologic tumor measurement based on computed tomography is routinely used to monitor patients. Both Tg biosynthesis and secretion are partially retained in dedifferentiating DTC cells. However, the synthesis and secretion rate is reduced (i.e., poor Tg secretors) in comparison to normal thyrocytes and well-differentiated cancer cells. Consequently, a large dedifferentiated tumor mass could be associated to low levels of circulating Tg, and, consequently, the role of serum Tg is rather limited in this scenario.

In a recent study on a small cohort of iodine-refractory patients with progressive disease undergoing treatment with lenvatinib, serum Tg fluctuations were frequently detected but do not necessarily reflect morphologic tumor alterations, especially shortly after lenvatinib dose reductions. However, whereas patients with controlled disease presented with oscillating tumor markers after an initial nadir without morphologic tumor progression, patients with true progression demonstrated a continuous rise in serum Tg [9]. Other authors also reported a decrease of Tg levels in most patients receiving TKIs, but neither baseline Tg nor Tg changes consistently correlated with the degree or duration of objective response [10].

A progressive dedifferentiation may be also observed in medullary thyroid carcinoma (MTC) cells, translating in a more aggressive tumor behavior especially in some of the patients with locally advanced or metastatic disease. As discussed in other sections of the present book, serum calcitonin (CT) and carcinoembryonic antigen (CEA) levels are related to tumor burden, though production of these markers may differ between tumors (i.e., tumors with low expression of calcitonin and higher production of CEA may be more aggressive) [11]. Nowadays, tyrosine kinase inhibitors (TKIs), especially vandetanib, have been recommended as first-line therapy in the case of aggressive metastatic MTC patients based on phase II and phase III trials in MTC patients that reported higher objective response rates compared with cytotoxic chemotherapy [12].

Indeed, no strict correlation has yet been reported between early changes in CT and CEA levels, and response to TKI and even paradoxical increase in biomarkers was observed in responders [13].

Overall, currently available results suggest that the mechanisms leading to tumor control and tumor marker Tg, CT, and CEA secretion are likely dissociated in the setting of TKI administration in patients with advanced radioiodine-refractory DTC and advanced MTC as well. As previously remarked, changes in serum Tg, CT, and CEA should always be confirmed by imaging in the setting of TKI treatment of advanced DTC and MTC, respectively. However, conventional imaging criteria (i.e., RECIST) may also have their own limitations when determining the effects of TKIs on tumor volume [14]. Therefore, new circulating biomarkers are warranted to help identify patients most likely to benefit from these therapies. Recently, among a series of candidate tumor markers, carbohydrate antigen 19-9 (CA 19-9) and cytokeratin fragments 19 (Cyfra 21.1) emerged as potentially useful prognostic predictors in both advanced DTC (CA 19-9 and Cyfra 21.1) and MTC (CA 19-9), respectively. Biology and physiopathology, assay methods and laboratory pitfalls, current clinical data, and potential applications of such tumor markers will be addressed in following sections.

12.2 Carbohydrate Antigen 19-9 (CA 19-9)

Mucins (MUCs) are heavily glycosylated, high molecular weight glycoproteins with an aberrant expression profile in various malignancies. So far 19 mucin genes have been described; eight of them are now well-characterized (i.e., *MUC1-4*, *MUC5B*, *MUC5AC*, *MUC6*, and *MUC7. MUC8, MUC9, MUC11,* and *MUC12*

have also been partially sequenced, but their characterization is yet to be completed, *MUC13* was identified as a cell surface mucin expressed by epithelial cells as well as hematopoietic cells, and *MUC16* was characterized from a partial cDNA sequence encoding a mucin that has long been known as the ovarian cancer marker CA125) [15]. Mucins are synthesized either as membrane-bound or as secreted glycoproteins by epithelial cells in the lungs, stomach, intestines, eyes, and several other organs. Under normal circumstances, they line the apical surface of epithelial cells and protect the body from infection by pathogen binding to oligosaccharides in the extracellular domain, preventing the pathogen from reaching the cell surface [16]. In addition, their involvement in the renewal and differentiation of the epithelium, modulation of cell adhesion, as well as cell signaling has also been proposed. Of relevance, alterations in the expression and in the structure of mucins have been reported in both preneoplastic and neoplastic lesions [17]. Mucin 1, cell surface-associated (MUC1) or polymorphic epithelial mucin (PEM), is a mucin encoded by the *MUC1* gene in humans. MUC1 is extensively O-linked glycosylated in its extracellular domain. MUC1 is overexpressed in colon, breast, ovarian, lung, and pancreatic cancer cells, and its associated glycans are shorter than those of nontumor-associated MUC1 [18]. Indeed, mucins were proved to be involved in dedifferentiation of tumor tissues and to promote resistance to treatments (Fig. 12.1).

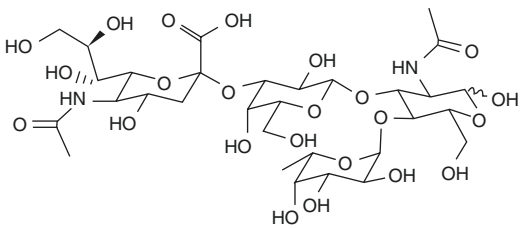

Fig. 12.1 Expression and release of CA 19-9 in cancer progression. Galli C et al. CA 19-9: handle with care Clin Chem Lab Med 2013;51(7):1369–1383 (permission obtained)

In fact, the heavy glycosylation in the extracellular domain of MUC1 creates a highly hydrophilic region which prevents the drugs from reaching their targets and allows cancer cells which produce a large amount of MUC1 to concentrate growth factors near their receptors, increasing receptor activity and the growth of cancer cells. MUC1 cytoplasmic tail has been also shown to bind p53 and to increase expression of Bcl-xL preventing both p53- and mitochondrial cytochrome c-mediated apoptosis. Additionally, MUC1 may prevent the interaction of immune cells with receptors on the cancer cell surface through steric hindrance. Finally, increased expression of MUC1 promotes cancer cell invasion through beta-catenin, resulting in the initiation of epithelial-mesenchymal transition which promotes the formation of metastases. The most commonly used clinical tests for mucins as tumor markers are serum-based immunoassays for blood group-related antigens and glycoproteins like CA 125, CA 15-3, CA 242, and CA 19-9. The CA 19-9 or carbohydrate antigen sialyl-Lewis is a frequently used tumor marker for cancers of the digestive tract. Although the maximum tissue expression of CA 19-9 occurs in pancreatic cancer, this antigen is not tissue specific, because it has been demonstrated in cancers involving other organs, such as the stomach, lung, colon, breast, ovary, and uterus [19]. Increase of this antigen expression in tissues and blood depends on the tumor-related hypoxia that, in turn, induces the transcription of several glycogenes involved in sialyl-Lewis a synthesis and is associated with a greater probability of the patient developing hematogenous metastasis. It has recently been reported that there is another form of the molecule, named *disialyl-Lewis a*, that is predominantly expressed in nonmalignant epithelial cells, connected to two sialic acid molecules. This molecule normally helps to maintain immune homeostasis of the gastrointestinal mucosa. In the early stages of carcinogenesis, inhibition of the sialyl-transferase gene causes a partial synthesis because of the incomplete connection of the second residue of sialic acid and the resulting accumulation of monosialyl Lewis

in cancer cells [20]. Although it was characterized almost three decades ago, according to the current international guidelines, based on available evidence, CA 19-9 is still the most commonly used serum tumor marker for the monitoring of pancreatic cancer: the levels should fall when the tumor is treated, and they may rise again if the disease recurs [21]. Thus far, the lack of specificity does not support its use for the diagnosis of the early forms of pancreatic cancer [20, 22], and its potential applications for the differential diagnosis are still debated. In fact, its sensitivity (70–80%) and specificity (68–91%) are not considered sufficient [21, 23]. Positive findings may occur in several situations other than malignant neoplasia such as patients with inflammatory processes (chronic and acute pancreatitis, cholangitis, and liver cirrhosis), characterized by high concentrations of CA 19-9 [24], which decrease to normal values after appropriate treatment. In these cases, the positivity is common for almost all methods. The increases of CA 19-9 in patients with benign disease are quite unavoidable, and the only current analytical approach to better define the nature of such elevations could be the assessment of the relationship between *sialyl-Lewis a* and *disialyl-Lewis* a, which is not elevated in malignant disease [25]. In addition, the finding of elevated CA 19-9 levels in healthy subjects for whom this test should not be ordered may have important implications from a psychological point of view with the requirement of many diagnostic tests to be performed [26, 27].

The dependence of tissue expression and circulating levels of *sialyl-Lewis a* on the Lewis blood group influences the sensitivity of testing for CA 19-9. In fact, false-negative results will always be found in subjects with *Lewis a*-negative genotype, representing 5–10% of the Caucasian population, whereas no data on other races are available [21]. Recently, it has been reported that low or medium (higher than 100 kU/L) levels of CA 19-9 may be found in some patients with *Lewis a*-negative genotype and suffering from advanced pancreatic cancer [28]. It was probably due to the situation of homozygosity for the secretory gene and overproduction of glycan precursors [29].

12.3 Measurement of Circulating CA 19-9

A commercial assay for CA 19-9 was developed in 1983 [30]. Radioimmunoassays were first used for the determination of CA 19-9 in the blood and other biological fluids, but they were quickly replaced by immunoassays (IMAs) [20] (Table 12.1).

Almost all of the IMAs for the quantitative detection of CA 19-9 utilize a sandwich assay format and depend on the use of the monoclonal antibody 1116-NS-19-9, named Centocor, that recognizes a sialylated lacto-N-fucopentaose II epitope occurring on the mucin, and it is related to the Lewis a blood group [20, 31] (Fig. 12.2).

The original hybridoma secreting the monoclonal antibody 1116-NS-19-9 was developed by immunizing mice with the SW1116 human cancer cells. The minimal structure recognized by this antibody is the terminal tetrasaccharide of the CA 19-9 antigen. Removal of the fucose residue or the sialic acid moiety cancels or decreases the antigen-antibody interaction [32, 33].

The interpretation of CA 19-9 results is often altered by nonspecific elevations both in diseased and healthy subjects, either because of associate morbidity (see above) or IMA interference, leading to misdiagnosis and further unnecessary and expensive examinations [33, 34].

In particular, similarly to other IMAs, also for the CA 19-9, different studies in the literature have reported cases of interference due to rheumatoid factor and heterophilic antibodies. The latter were responsible for 44.4% of the discrepancies observed between two automated IMAs for CA 19-9 as reported by Passerini et al. in a recent paper [33, 35, 36].

Rheumatoid factor and heterophilic antibodies are endogenous autoantibodies found in serum/plasma, mainly of IgM class, that can bind to immunoglobulins (preferably IgG) of other species. Thus, they usually affect the "sandwich" assay by bridging the capture and detection antibodies causing an increased signal and consequently a falsely elevated measured concentration [37]. A nonlinear response to dilution is suggestive of antibody interference. However, it must be

Table 12.1 Analytical performance characteristics of the main current CA-19.9 automated immunoassays (Manufacturers' data)

Manufacturer	Analyzer	Methodology	Assay principle/tracer	Monoclonal antibodies	Imprecision (CV): intra-; inter-; total (%)	LoD (kU/L)	Assay range (kU/L)	Cutoff (kU/L)	High-dose Hook effect (kU/L)
Abbott Diagnostics	ARCHITECT	CMIA	Noncompetitive; heterogeneous/ acridinium esters	1116-NS-19-9	2.3–8.0; nd; 3.4–8.5	2.0	0–1200	37	> 1,750,000
Beckman Coulter	Access	CLIA	Noncompetitive; heterogeneous/ Lumi-Phos 530	Anti-CA 19-9 antibodies	1.7–6.4; 2.4–5.7; 3.0–8.9	0.8	0.8–2000	35	> 800,000
bioMerieux	VIDAS 3	ELFA	Noncompetitive; heterogeneous/4MUP	1116-NS-19-9	2.2–2.9; 2.5–4.2; nd	3	3–500	37	> 1000,000
Thermo Fisher Scientific BRAHMS	Kryptor	FEIA (TRACE)	Noncompetitive; homogeneous/ europium cryptate-XL665	1116-NS-19-9	1.4–3.0; 4.8–5.9; nd	1.2	1.2–700	37	> 600,000
Diasorin	Liaison	CLIA	Noncompetitive; heterogeneous/ isoluminol derivatives	1116-NS-19-9	1.9–10.0; 4.9–7.5; nd	0.3	0.3–1000	37	> 1,550,000
Fujirebio	Lumipulse G	CLEIA	Noncompetitive; heterogeneous/ AMPPD	1116-NS-19-9	0.8–1.2; nd; 1.5–3.4	0.894	2–500	37	nd
Ortho Clinical Diagnostics	Vitros	CLIA	Noncompetitive; heterogeneous/ luminol derivatives	1116-NS-19-9	0.8–1.4; nd; 2.6–5.7	1.4	1.4–1000	37	> 965,000
Roche Diagnostics	Cobas/Elecsys	ECLIA	Noncompetitive; heterogeneous/ ruthenium derivatives	1116-NS-19-9	1.2–4.4; 1.9–8.0; nd	0.6	0.6–1000	34	> 500,000
Siemens Healthineers	Advia Centaur	CLIA	Noncompetitive; heterogeneous/ acridinium esters	1116-NS-19-9	3.4–10.4; 2.4–5.3; 4.3–11.7	1.2	1.2–700	37	> 5,800,000
Siemens Healthineers	Advia Dimension Vista	CLIA (LOCI)	Noncompetitive; homogeneous/ Phthalocyanine-olefin	1116-NS-19-9	1.1–5.8; 2.7–8.9; nd	2.0	2–1000	37	> 1000,000
Siemens Healthineers	Immulite XPi	CLIA	Noncompetitive; heterogeneous/ adamantyl dioxetane phosphate	Anti-CA 19-9 antibodies	4.2–7.5; 5.5–6.9; nd	1.0	1–1000	18.4	> 50,000
Snibe	Maglumi	CLIA	Noncompetitive; heterogeneous/ ABEI	Anti-CA 19-9 antibodies	4.76–6.04; 8.52–9.64; nd	1.0	1–1000	37	> 10,000

(continued)

Table 12.1 (continued)

Manufacturer	Analyzer	Methodology	Assay principle/tracer	Monoclonal antibodies	Imprecision (CV): intra-; inter-; total (%)	LoD (kU/L)	Assay range (kU/L)	Cutoff (kU/L)	High-dose Hook effect (kU/L)
Tosoh Bioscience	AIA	FEIA	Noncompetitive; heterogeneous/4MUP	Anti-CA 19-9 antibodies	2.3–2.8; nd; 3.9–4.4	1.0	1–400	37	> 200,000
Tosoh Bioscience	AIA CL2400	CLEIA	Noncompetitive; heterogeneous/ DIFURAT®	Anti-CA 19-9 antibodies	3.6–4.5; 3.9–5.1; nd	0.17	0.5–1500	34.4[a] 37.7[b]	nd

4MUP 4-methyl-umbelliferyl phosphate, *ABEI* N-(aminobutil)-N-(ethyl)-isoluminol, *CLIA* chemiluminescence immunoassay, *AMPPD* alkaline phosphatase-spiroadamantyl-methoxy-phosphoryloxy-phenyl-dioxetane, *CLEIA* chemiluminescence enzyme immunoassay, *CMIA* chemiluminescent microparticle immunoassay, *DIFURAT®* 3-(5-tert-Butyl-4,4-dimethyl-2,6,7-trioxabicyclo[3.2.0]hept-1-yl) phenylphosphate disodium salt, *ECLIA* electrochemiluminescence immunoassay, *ELFA* enzyme-linked fluorescence assay, *FEIA* fluorescence enzyme immunoassay, *LOCI* luminescent oxygen channeling immunoassay technology, *LoD* limit of detection, *nd* not declared

[a]Defined in Asian population

[b]Defined in Caucasian population

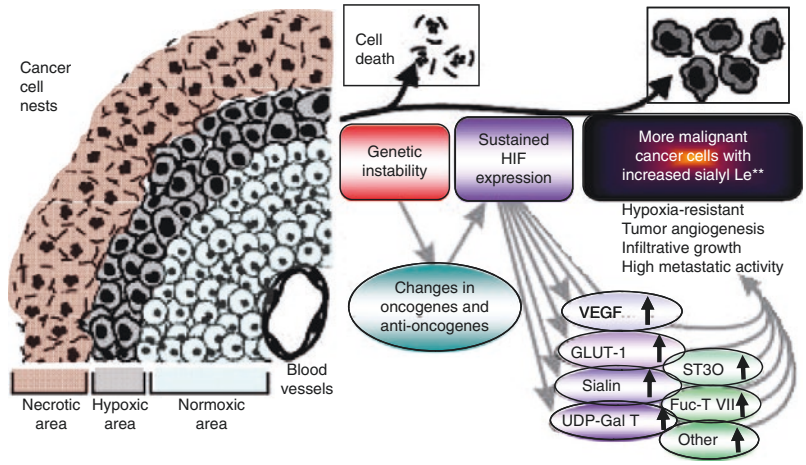

Fig. 12.2 Chemical structure of the sialyl-Lewis *a* determinant. Galli C et al. CA 19-9: handle with care Clin Chem Lab Med 2013; 51(7): 1369–1383 (permission obtained). *FUC-T-VII* fucosyltransferase VII, *GLUT-1* glucose transporter, *HIF* hypoxia-inducible factor, *Sialin sialic* acid transporter, *ST3O* sialyl-transferase, *UDP-GALT* UDP galactose transporter, *VEFG* vascular endothelial growth factor

noted that a nonlinear response can also result from the hook effect or cross-reactivity. Moreover, nonlinear dilution, with increased recovery of the antigen, is common in IMAs for mucins. This is probably due to a variety of factors (i.e., the inherent property of mucins to aggregate and disaggregate into a range of molecules species, the presence of anti-carbohydrates, and of other not well-known matrix-related effects, etc.) [32]. Therefore, it has been suggested that nonlinear response to dilution may not be an appropriate method to detect interference [32, 38].

All in all, the laboratory should be aware of IMA interference and should apply a systematic approach in the investigation of such phenomenon: first, the repetition of the analysis to confirm the result and then, if possible, the use of an alternative IMA to inquire about the discrepancy. Of course, the close interaction between the laboratory and the physicians is essential [36, 39].

An additional important limitation of CA 19-9 determination is the particular sensitivity of the assay to viral and bacterial neuraminidases resulting in false negatives. Thus, samples should be carefully prepared to avoid bacterial contamination [32].

The interpretation of CA 19-9 results is also made difficult by the inter-method discrepancy showing significant CA 19-9 disparities in the same individual samples. In fact, the studies performed in the last decade focusing on this issue reported similar data and reached analogous conclusions: the recent automation of IMAs has certainly improved the assay imprecision but has also helped to impair the concordance between the results obtained with different methods [40–46].

Another relevant aspect related to the CA 19-9 analytical specificity and the recurrent inter-method discrepancy is the concept of threshold/cutoff. Undoubtedly, the cutoff for CA 19-9 depends on the background: no values can be suggested to strictly distinguish between benign and malignant disease. Moreover, the upper reference limit is usually established at the 95th or 97.5th percentile in a healthy reference sample, not representing the "real-life" sample that will be tested for this biomarker. Finally, it would be important considering ethnic diversities in the reference population [41, 42]. All in all, the authors concluded their papers on this issue suggesting the use of the same method for the monitoring of patients with cancer and inviting laboratories to indicate in the report the name of the method employed [20]. Taken into consideration the aforementioned aspects, the scientific societies and the manufacturers should work together in order to improve CA 19-9 harmonization, making available an international reference material and following existing programs for method assessment and correction of bias, as was the case with other IMAs. In fact, the differences between methods are attributable to numerous variables involved in IMAs such as assay technology, reaction kinetics, incubation times,

dilution, and, overall, the use of different antibodies [20, 44]. With regard to this last point, Partyka et al. [47] have recently demonstrated the improvement of cancer detection by using the antibody with broader specificity beyond the sialyl-Lewis a antigen, suggesting that the additional glycans were also elevated in a cancer-specific manner. All in all, Partyka's analysis was useful for understanding the factors that may further improve upon the CA 19-9 assay, suggesting that the use of different antibodies can lead to a better sensitivity in patients with malignant neoplasia without an elevation of reactivity in non-malignant disease.

12.4 CA 19-9 and Thyroid Cancer

The natural history of medullary thyroid cancer (MTC) varies from rapid progression and survival over a few years to a very slow progression, or stable disease, that extends for decades. Overall, the prognosis of MTC patients is related to the extent of the disease at the time of diagnosis (i.e., median 5-year survival rate is 50% in the presence of distant metastases). As discussed in other section of the present book, calcitonin and procalcitonin are very sensitive and specific serum marker for the diagnosis of MTC, and carcinoembryonic antigen (CEA) is also employed while monitoring MTC patients. In 2011, Milman and colleagues [48] described the case of a 56-year-old woman with multiple endocrine neoplasia 2B syndrome presented with extensive metastatic spread of MTC to the lungs and liver, 47 years after the original diagnosis. The patient's calcitonin level decreased from 2950 to 261 pg/mL over a 20-year period. The serum CEA level was elevated at 6800 ng/mL; serum CA 19-9 and CA 125 tumor markers were also measured and found to be significantly elevated, at 39,334 U/mL and 96.2 U/mL, respectively. Immunostaining of the metastatic MTC tissue showed patchy staining for calcitonin, strongly positive staining for CEA and CA 19-9, and weakly positive staining for CA 125. Basing on this picture, they postulated that high serum levels of CA 19-9 could be considered a marker of MTC dedifferentiation and disease aggressiveness. Two years later, Elisei and colleagues [49] reported the peculiar case of a young patient with MEN 2A who rapidly died from aggressive MTC 10 months after initial diagnosis. Her CA 19-9 increased up to >10,000 IU/mL, and immunohistochemistry of the thyroid nodule was performed at the autopsy and demonstrated positive staining for CT and CA 19-9 in the primary tumor. Then, the same group measured CA 19-9 in 100 advanced structural recurrent/persistent MTC patients and in 100 MTC patients cured or with biochemical but structural disease [50]. Sixteen percent of patients with advanced diseases had high CA 19-9 and concomitantly higher levels of CEA and CT compared with the group with normal CA 19-9 levels. None of patients with controlled disease had high CA 19-9 levels; moreover, among patients with advanced disease those with high CA 19-9 levels showed a higher mortality rate than patients with normal CA 19-9 serum levels. Overall, these results demonstrated that increased CA 19-9 levels in serum is an adverse prognostic factor in patients with advanced MTC and identifies those cases with a higher risk of short-term mortality. Recently, Milman and colleagues [51] evaluated whether positive CA 19-9 staining of primary MTC tissue predicts metastatic potential. Among specimens from 16 patients, 63% stained positive for CA 19-9; indeed, all specimens from patients with advanced (i.e., stage IV) MTC stained positive for CA 19-9, compared to only 40% of cases with stages I to III. Importantly, 100% of the primary specimen with associated metastatic spread over time stained positive for CA 19-9. As a consequence, a negative CA 19-9 staining excludes a stage IV MTC with a 100% negative predictive value.

Similarly, serum CA 19-9 levels were reported to be elevated in some cases of anaplastic thyroid carcinoma and in papillary thyroid carcinomas with poor differentiated features, aggressive tumor behavior, and a worse prognosis [52].

12.5 Cytokeratin Fragment 19 (Cyfra 21.1)

The cytoskeleton of eukaryotic cells is responsible for the mechanical integrity of the cell and is a critical participant in several cellular

processes, such as cell division, motility, and cell/cell contact. It is composed of three different types of distinct filamentous structures: microfilaments, intermediate filaments (IF), and microtubules [53].

The IF protein family includes several hundred of different members. In turn, these are classified basing on structural similarities. Intermediate filament types I and II constitute the cytokeratins (acidic and basic proteins, respectively). The type III group includes desmin, vimentin, and glial fibrillary acidic proteins; type IV includes the neurofilament proteins (NF-L, NF-M, and NF-H) and internexin, while type V proteins are known as nuclear lamins, exclusive to the cell nuclei. The remaining IF proteins, sometimes called type VI, include filensin and phakinin [54].

The expression of cytokeratins varies with epithelial cell type, extent of differentiation, and development of the tissue. During the transformation of normal cells into malignant cells, the cytokeratin patterns are usually maintained, and this property has enabled cytokeratins to be applied as tumor markers [55, 56]. In the cytoskeleton, cytokeratins demonstrate poor solubility, but when present in the circulation, cytokeratins are detected either as partially degraded single protein fragments, as small complexes, or as large polymeric protein complexes, while intact, nondegraded, cytokeratins have not yet been detected in the bloodstream. The release of soluble cytokeratin fragments into the circulation involves multiple pathways including proteolytic degradation of cytokeratins in dying cells, abnormal mitosis, spillover of monomeric cytokeratin polypeptides from proliferating cells, apoptosis, and neoangiogenesis. Upon release from the tumor cells, cytokeratins can be detected in blood as well as in other body fluids. In normal, apparently healthy individuals, the level of cytokeratins in the circulation is low. However, levels rise significantly in patients with epithelial cell-associated carcinomas. Stratified squamous epithelia express mostly cytokeratins 1–6 and 9–17, while cytokeratins 7, 8, and 18–20 are identified in simple epithelia. Of the latter, cytokeratins 8, 18, and 19 are the most abundant ones in malignancy [57]. The most widely applied cytokeratin tests use the monoclonal-based assay tissue polypeptide antigen (TPA), cytokeratin fragment 19 (Cyfra 21.1), and tissue polypeptide-specific antigen (TPS). TPA (tissue polypeptide antigen) measures cytokeratins 8, 18, and 19 in serum samples [58] and is an example of a broad-spectrum cytokeratin assay demonstrating high sensitivity in cancer patients with various epithelial cell-associated carcinomas such as breast cancer, colorectal cancer, lung cancer, head and neck cancer, and bladder cancer [59–63].

The TPS is a specific cytokeratin-based assay, which detects a defined epitope structure on human cytokeratin 18 using the M3 monoclonal antibody. It was evaluated and proposed in various epithelial cell-associated carcinomas such as breast cancer, ovarian cancer, prostate cancer, and gastrointestinal cancer [64–66]. Finally, an assay measuring soluble cytokeratin 19 fragments in the circulation, Cyfra 21.1, exemplifies a monospecific cytokeratin assay [67]. Unlike the majority of epitopes, detectable by useful tumor markers such as CEA, CA 15-3, and CA 19-9, which are glycoproteins, Cyfra 21.1 is unique in the fact that its epitope is a polypeptide, probably released as a result of cell death [68].

Most reports in the literature have focused on the clinical use of Cyfra 21.1 in lung cancer and in head and neck cancer [69–71]. Although based on detection of the same type of proteins in serum, the individual cytokeratin immunoassays may give different profiles of reactivity likely due to the different detector antibodies employed and the different release of cytokeratin fragments into the circulation from one cytokeratin to another. All in all, as with most tumor markers, the cytokeratin assays are not interchangeable.

12.6 Measurement of Circulating Cyfra 21.1

Five decades ago, for the first time, two IMAs for the measurement of Cyfra 21.1 were introduced: a two-site sandwich immunoenzymometric assay (IEMA) and a two-site sandwich immunoradiometric assay (IRMA), respectively [68, 72].

These methods used two mouse monoclonal antibodies (KS 19-1 and BM 19-21) directed against two different epitopes of a fragment of cytokeratin 19, which is referred to as serum Cyfra 21.1. The target sites of the two monoclonal antibodies lie within amino acids 346–367 for BM 19.21 and 311–335 for KS 19.1: both epitopes are located in C-terminal helical domain of the molecule. These monoclonal antibodies were obtained by immunization against the MCF-7, a breast cancer cell line. Afterward, a large number of other automated methods have been developed, including monoplex immunoassays, electrochemiluminescence immunoassay (ECLIA) [73–78], chemiluminescent microparticle immunoassay (CMIA) [79, 80], heterogeneous chemiluminescent immunoassay (CLIA) [81], chemiluminescent enzyme immunoassay (CLEIA) [45, 82, 83], and luminescent proximity oxygen channeling immunoassay (LOCI) [84], and multiplex immunoassays, addressed laser bead immunoassay (ALBIA) [85] and lateral flow immunoassay (LFIA) [86]. All these recent automated methods are based on the same principle of the first IMAs; in particular, they are heterogeneous and noncompetitive, "two-step" sandwich, automated or automatable, and characterized by the use of two monoclonal antibodies: the first with acceptor function (KS 19.1), prevalently coated of a solid phase (paramagnetic microparticles, iron beads, streptavidin-coated microparticles, magnetic microbeads coated with anti-FITC, beads coated with fluorophores, nitrocellulose membranes, etc.) and the second (BM 19.21), with tracer function, coated to fluorophores (europium cryptate, phycoerythrin) or luminescent molecules (acridinium esters, alkaline phosphatase-spiroadamantyl-methoxy-phosphoryloxy-phenyl-dioxetane, ruthenium derivatives, N-(aminobutil)-N-(ethyl)-isoluminol, phthalocyanine-olefin, etc.) or enzymes (alkaline phosphatase, etc.). In addition, these methods show good analytical performances in terms of sensitivity (the LoD is in general very low, ranging from 0.01 to 0.20 μg/L), specificity (no critical pre-analytical phases, no interferences with other analytes, Hook effect at very high concentrations, etc.), precision (intra-assay <3.0% and inter-assay <6.0%), and accuracy (good correlation between different methods). For the aforementioned reasons, the upper reference limits are quite similar between methods, ranging from 1.5 to 5.4 μg/L (Table 12.2).

12.7 Cyfra 21.1 and Thyroid Cancer

The cytokeratin 19 (CK19) is an acidic protein that is part of the cytoskeleton of epithelial cells. Tissue CK19 is highly expressed in DTC, mainly those with papillary histotype (PTC) [87]. Increased preoperative Cyfra 21.1 levels were found in patients with localized aggressive histotypes of primary epithelial thyroid cancers, while they are usually normal in patients with primary and metastatic classical DTC histotypes [88]. More recently, it was demonstrated that patients with ^{131}I-refractory DTC metastases had significantly higher serum Cyfra 21.1 levels than patients with ^{131}I-avid ones. Such differences argue that ^{131}I-refractory thyroid cancer cells (i.e., dedifferentiated cells) are likely the source of the increased serum Cyfra 21.1 [89]. No data are currently available on the relationship between serum and tissue Cyfra 21.1 expression in DTC; however, increased serum Cyfra 21.1 levels were previously reported in patients with primary aggressive thyroid carcinomas despite low or absent CK19 immunostaining in corresponding tumor tissues [90]. Previous studies in human lung and liver cancer cell lines showed that among CK19-producing cells, only those with caspase-3 (an enzyme involved in apoptosis phenomena) expression induced high Cyfra 21.1 levels in culture supernatants [91–93]. Indeed, serum caspase-3 enzyme activity is detectable in patients with metastatic^{131}I-refractory thyroid cancer [94].

Globally, thyroid tumors with high proliferation rate, diffuse apoptosis, and necrosis are likely to release Cyfra 21.1 via caspase-3 action. The fast processing of CK19 molecules may explain the coexistence of a negative tissue CK19 staining with high levels of CK19-soluble fragments in serum of patients with such

Table 12.2 Analytical performance characteristics of the current Cyfra 21.1 automated immunoassays

• Manufacturer	Analyzer	Methodology	Assay principle/tracer	Monoclonal antibodies	Imprecision (CV): intra-; inter- (%)	LoD (μg/L)	Assay range (μg/L)	Cutoff (μg/L)	High-dose Hook effect (μg/L	Ref
Abbott Diagnostics	**ARCHITECT**	CMIA	Noncompetitive; heterogeneous/acridinium esters	BM 19.21 – KS 19.1	1.0–5.0; nd; 2.0–6.0[a]	0.09	0.5–100	2.1	750	Manufacturer data
Thermo Fisher Scientific BRAHMS	**Kryptor**	FEIA (TRACE)	Noncompetitive; homogeneous/europium cryptate and XL 665	BM 19.21 – KS 19.1	1.7–3.6; nd	0.16	0.16–350	3.3	> 4000	Manufacturer data
Fujirebio	**Lumipulse**	CLEIA	Noncompetitive; heterogeneous AMPPD	BM 19.21 – KS 19.1	0.6–2.1; 2.7–3.5	0.01	0.5–100	1.5	nd	Patel, 2010
Roche Diagnostics	**Cobas/Elecsys**	ECLIA	Noncompetitive; heterogeneous ruthenium derivatives	BM 19.21 – KS 19.1	1.1–2.1; 2.8–3.3	0.20	0.1–500	5.4	> 2000	Sanchez-Carbayo, 1999
Snibe	**Maglumi**	CLIA	Noncompetitive; heterogeneous ABEI	Anti-Cyfra 21.1 antibodies	5.4–6.1; 8.3–9.8	0.20	0–600	2.7	> 12,000	Lumachi, 2014
Perkin-Elmer	**Alphalisa**	CLIA (LOCI)	Noncompetitive; homogeneous Phthalocyanine-olefin	Anti-Cyfra 21.1 antibodies	3.4–9.0; 4.0–10.0	0.08	0–500	nd	nd	He, 2013
Luminex	**MAGPIX**	FEIA	Multiplex immunoassay Phycoerythrin	BM 19.21 – KS 19.1	3.1–8.1; 2.6–14.1	nd	nd	nd	nd	Doseeva, 2015
nd	**aQcare TRF**	LFIA	Multiplex immunoassay Quantum-dot	Anti-Cyfra 21.1 antibodies	5.0–9.0; 6.9–9.6	0.16	0–480	nd	> 480	Chen, 2017

4MUP 4-methyl-umbelliferyl phosphate, *ABEI* N-(aminobutil)-N-(ethyl)-isoluminol, *CLIA* chemiluminescence immunoassay, *AMPPD* alkaline phosphatase-spiroadamantyl-methoxy-phosphoryloxy-phenyl-dioxetane, *CLEIA* chemiluminescence enzyme immunoassay, *ECLIA* electrochemiluminescence immunoassay, *FEIA* fluorescence enzyme immunoassay, *LOCI* luminescent oxygen channeling immunoassay technology, *LFIA* later flow immunoassay, *LoD* limit of detection, *nd* not declared

[a]Total imprecision

aggressive thyroid tumors [89, 90, 95]. Vice versa, low proliferation rate and absent of apoptosis phenomena explain low serum levels of Cyfra 21.1 in patients with classical DTC [88, 90]. Interestingly, high Cyfra 21.1 levels were found in ^{131}I-refractory patients even after exclusion of those patients with primary aggressive thyroid carcinomas. This is in line with previous reported differences between primary differentiated thyroid carcinomas and their metastases at the genetic level, as the number of chromosomal abnormalities increases as thyroid carcinomas progress [96]. Then, although the majority of primary thyroid carcinomas leading to ^{131}I-refractory disease were aggressive follicular and papillary histotypes, primarily well-differentiated tumors may be also responsible for ^{131}I-resistance and increased Cyfra 21.1 levels. As previously remarked, serum Tg measurement and RECIST assessment have their own limitations when determining the effects of TKIs. Therefore, new circulating biomarkers are warranted to help identify patients most likely to benefit from these therapies. Even if prospective randomized studies will be designed to independently validate its predictive and/or prognostic, serum Cyfra 21.1 may serve as a marker for recurrent ^{131}I-refractory thyroid cancer and is an important potential monitoring tool for alternative treatment approaches.

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