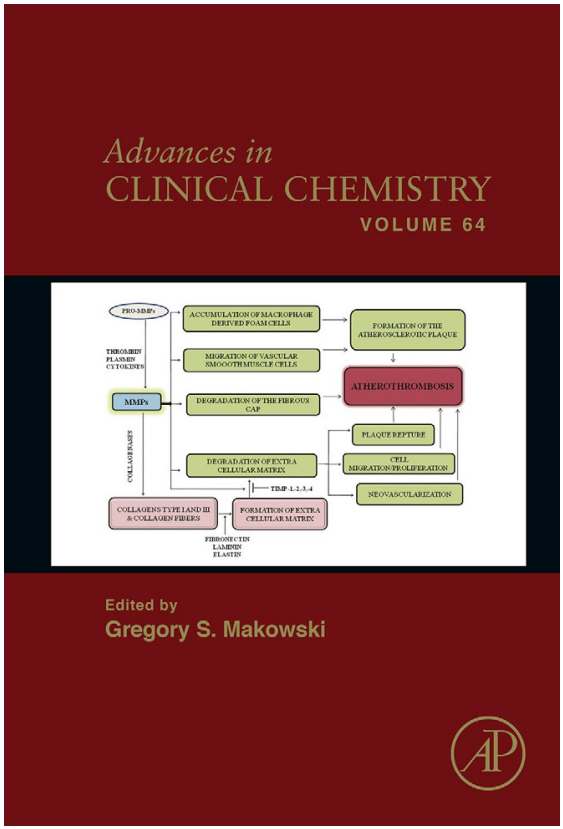

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CHAPTER SIX

Mammaglobin A: Review and Clinical Utility

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Contents

Abstract

Mammaglobin A is a protein that belongs to the secretoglobin superfamily. It has highly specific expression in cells from most breast cancers and may be used to detect circulating or disseminated tumor cells. In addition, mammaglobin A is currently under investigation as a potential therapeutic target for immune therapies that target breast cancer. The present review will highlight our current understanding of mammaglobin A at the genetic and protein level and its potential clinical applications. Characteristics of breast cancer and methods used to isolate and detect circulating tumor cells will also be presented.

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1. MAMMAGLOBIN A: GENE AND PROTEIN STRUCTURE

In 1994, Watson and Fleming isolated complementary DNA fragments corresponding to mRNA of an unknown protein whose expression was increased in breast cancer biopsies versus normal breast tissue controls [1]. Two years later, the same authors reported the complete cDNA of the protein isolated from mammary adenocarcinoma [2]. They called this new gene human mammaglobin or mammaglobin A.

Sequence analysis has demonstrated that the 93 amino acid polypeptide encoded by the mammaglobin cDNA is homologous to a family of proteins that include rat prostatic steroid binding protein (prostatein) subunit C3 (rPSC3) (~42% homology) [3], rabbit uteroglobin (rUg) [4], the Clara cell 10 kDa protein (CC10) from multiple species [5], and the Clara cell 16 kDa protein (CC16). All these proteins belong to the gene family of the uteroglobin/CC10, that is, the secretoglobin superfamily [6–8]. The suffix "globin" refers to hemoglobin and has a structural meaning in the term globin-fold, which is a bundle of eight alpha-helices connected by rather short loop regions and arranged so that the helices form a pocket for binding of the heme group. Similarly, each monomer of the secretoglobin family of proteins is a four-helix bundle, and the dimer (an eight-helix bundle) forms a pocket for the binding of numerous hydrophobic molecules [7].

The number of members of the secretoglobin superfamily has been growing over the last few years, and these proteins have been found in both mammals and birds [9,10]. Currently, this family includes 10 human secretoglobins genes and 5 pseudogenes (Table 6.1) [8,11].

The secretoglobins are small rarely glycosylated proteins that form homodimers, heterodimers, and heterotetramers [11]. Secretoglobins are mainly expressed in mucosal tissues and are found at high levels in many secretions including those from uterine, prostatic, pulmonary, and lacrimal and salivary glands. Little is known about their physiologic role, although some of them have been implicated in lung maintenance and repair, cancer development, immune system regulation, chemotaxis, and possibly the transport of aromatic molecules like steroid hormones and biphenyls [8,12–14].

Genes that encode mammaglobin A and most of the other members of the human secretoglobin superfamily are located on chromosome 11q12.3–13.1 where they are clustered. Based on their structure, they

Table 6.1 Human secretoglobins genes

Common protein names	Approved symbol	Other names
Uteroglobin	SCGB1**A1**	Clara cell 16-kDa protein (CC16)
Mammaglobin A	SCGB2**A2**	Mammaglobin 1, hMAM, mammaglobin
Mammaglobin B	SCGB2**A1**	Mammaglobin 2, MGB2, lipophilin C
Lipophilin A	SCGB1**D1**	
Lipophilin B	SCGB1**D2**	BU101
Secretoglobin inducible by gamma-interferon	SCGB1**D4**	IFN-gamma inducible SCGB
Ligand-binding protein RYD5	SCGB1**C1**	RYD5
Pneumo secretory protein	SCGB3**A2**	Uteroglobin-related protein 1
Cytokine high in normal-1	SCGB3**A1**	Pneumo secretory protein 2
Secretoglobin-like protein	SCGB2**B2**	SCGBL

SCGB, secretoglobin (e.g., SCGB1**A1**: secretoglobin family 1A, member 1).

constitute a multigene family derived from a common ancestral sequence [6,9] (Fig. 6.1). This region is commonly amplified in breast carcinomas [15].

The mammaglobin A gene is composed of three exons and two introns [6]. This exon/intron arrangement and the corresponding splice sites are well conserved among secretoglobin family members. Sequencing of the mammaglobin A gene also revealed a number of repetitive sequences including a human-specific *alu* repeat motif in the 5′-nontranscribed region. An element reminiscent of a classical estrogen response element is located within the human-specific alu repeat sequence. A second element, which is similar to a functional androgen response element in the rPSC3(I) gene, is also located in the 3′-nontranscribed region [6,16].

A reporter gene was used to study the regulation of mammaglobin A expression in mammary carcinoma cells [17]. Results indicated that vectors carrying the human mammaglobin A promoter induced the expression of a reporter gene in mammary carcinoma cells at levels that were orders of

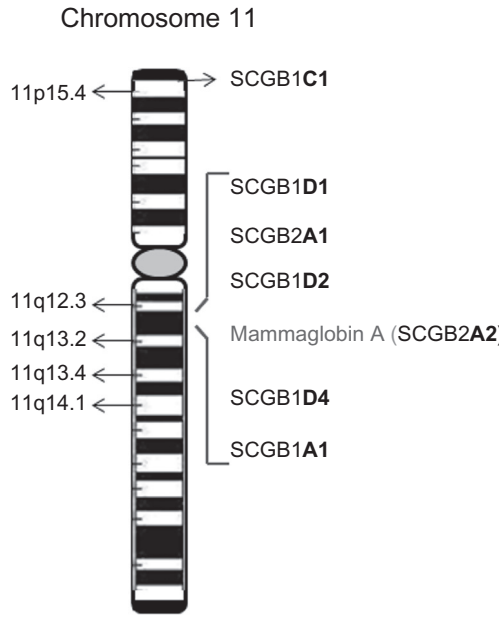

Figure 6.1 The graph shows the localization of most of the members of the human secretoglobin superfamily, including mammaglobin A, on chromosome 11q12.3–13.1.

magnitude higher than normal nonmammary cell types. A high degree of specificity was retained by promoter fragments as small as 344-bp, although the magnitude of expression was reduced 100-fold compared to the full-length promoter fragment of 8-kb. Deletion analysis indicated that a strong enhancer may be responsible for the increased expression of the full-length promoter relative to the 344-bp minimal promoter [17].

Mammaglobin A cDNA encodes a polypeptide of 93 amino acids with a molecular weight (Mw) of 10.5 kDa. The first 19 residues correspond to a hydrophobic signal peptide which is cleaved during the secretion of the mature 8.5-kDa protein [18]. In breast tissue, mammaglobin A is glycosylated and covalently bound to lipophilin B as a heterodimer in antiparallel fashion which allows formation of three disulfide bridges between the two molecules. At the same time, a heterotetramer of 45-kDa can be formed by noncovalent union of two heterodimers [14,18,19]. There are two N-linked glycosylation sites in the primary sequence of mammaglobin A and each site has an attached sugar of ~3500 Da [11].

Different mammaglobin A forms (18- and 25-kDa) were also detected in normal breast and breast cancer tissues [20]. The latter is likely the fully

glycosylated mammaglobin A–lipophilin B complex, while the former is likely a partially glycosylated mammaglobin A–lipophilin B complex.

Secreted mammaglobin A protein can be found as 14- to 21-kDa species that are likely products of posttranslational processing of its predicted 8-kDa size [21]. Using an enzyme-linked immunsorbent assay (ELISA), no significant differences were found in serum mammaglobin A concentration in pre- (7.90 ± 1.0 ng/mL) and postmenopausal (7.52 ± 0.72 ng/mL) women. Statistically, a mammaglobin A 8.8 ng/mL cutoff resulted in 68.8% sensitivity and 88.8% specificity for detecting breast cancer [21].

Zuo *et al.* [22] proposed that at least some mammaglobin A was associated with cell membranes due to the presence of mostly hydrophobic amino acids in its N-terminus (9th–18th residues), that is, a transmembrane helix. In the same study, mammaglobin A was variably detected (22–64%) on the membrane fraction of breast cancer cells but was dependent on cell type. Mammaglobin A was localized to the surface and cytoplasm of both normal and breast cancer cells. Interestingly, not all breast cancers or normal breast cells demonstrated mammaglobin A immunologically on their membranes. At present, the biologic role of mammaglobin A remains unknown.

2. EXPRESSION OF MAMAGLOBIN A IN HEALTHY TISSUE AND PRIMARY TUMORS

Using RT-PCR and Northern blotting, Watson and Fleming analyzed mammaglobin A mRNA expression in a variety of fetal and adult human tissues including breast, lung, salivary gland, uterus, prostate, ovary, testis, placenta, peripheral blood leukocytes, lymph nodes, and bone marrow. The authors showed that the expression of this protein was mainly restricted to the adult mammary gland [2,23,24]. It has been suggested that breast tissue mammaglobin A expression was not be associated with breastfeeding. Instead, mammaglobin A expression appeared related with the proliferation and terminal differentiation of the mammary gland [6,9].

Subsequent studies confirmed mammaglobin A overexpression in breast tumor [2,25]. Overexpression was observed in ~80% of breast tumors and was not associated with breast carcinogenesis [26]. In addition, results from different studies suggested that increased mammaglobin A expression in breast tumors was associated with clinical and biologic features characteristic of a less aggressive phenotype being significantly higher in estrogen-receptor-positive tumors [27,28].

In recent studies, mammaglobin A has also been detected in normal and malignant tissues of the female genital tract (endometrium, cervix, and ovary) and in malignant gynecologic effusion [9,29–34]. However, Grünewald *et al.* [30] found that mammaglobin A expression was significantly increased in breast relative to ovarian and endometrial tissue. It has been shown that mammaglobin A gene expression in endometrium was upregulated during implantation versus the late proliferative phase [35]. A recent study indicated that the expression of the protein was controlled by steroid hormones in normal human endometrium with peak expression during the luteal phase [36].

It has also been reported that mammaglobin A expression was detected in normal and tumor tissues of sweat and salivary glands and was absent or rarely expressed in other tissues and tumors [9,32,37].

Based on its highly specific expression in breast cancer, it was suggested that mammaglobin A could identify circulating tumor cells (CTC), disseminated tumor cells (DTC) and confirm a breast origin of metastatic cancer.

In order to understand the clinical usefulness of mammaglobin A, a brief review about cancer and methods used for detection of cancer cells will be presented in the following section.

3. CANCER AND METASTASIS

Sporadic cancer arises primarily from somatic cell mutations occurring from genetic disorders that accumulate over time. Carcinogenesis in humans is a multistep process and is age dependent. Uncontrolled cell proliferation is a major feature of cancer where tumor cells have acquired alterations in genes that are directly involved in regulating the cell cycle [38]. Cancer can develop the process of metastasis produced by a sequelae of related events involving multiple host–tumor interactions [39]. Metastasis is defined as the dissemination of neoplastic cells to nearby or distant secondary sites where they proliferate to form an extravascular mass of tumor cells [40].

Clonal selection takes place in the tumor, thereby generating a completely distinct genetic cell population having unique invasive characteristics and metastatic potential. The metastatic process involves several steps, all of which must be successfully completed to give rise to a metastatic tumor (Fig. 6.2). In this scenario, the primary tumor grows and develops new blood vessels to support its metabolic needs (angiogenesis).

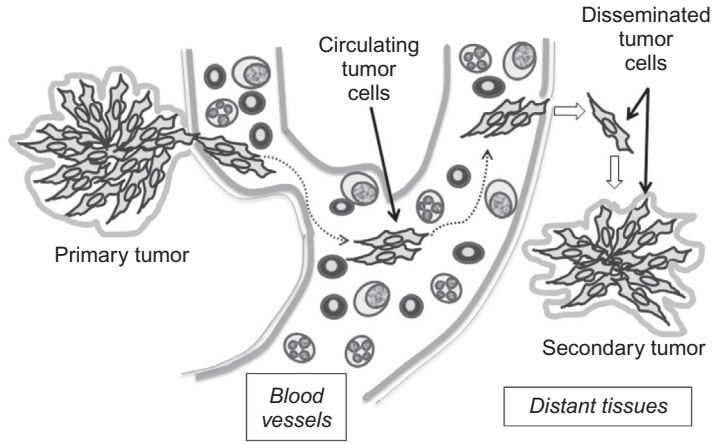

Figure 6.2 Scheme of the metastatic process. The cells from the primary tumor enter the bloodstream (or lymph), where they are called circulating tumor cells (CTC). Some of these CTC could extravasate and colonize a distant tissue, where they are known as disseminated tumor cells (DTC). The DTC could proliferate and form a secondary tumor (metastasis).

Tumor cells can leave the tumor and enter the circulatory system directly or via the lymphatic system (intravasation). CTC need to survive in the circulation until they extravasate from the circulation into the surrounding tissue. Cells that invade a new tissue must initiate and maintain growth to form metastases (secondary tumor), sustained by development of new blood vessels [41,42].

In each stage, the cell must evade the immune response and adverse metabolic conditions. Therefore, it is estimated that only one per 10^5–10^6 disseminated neoplastic cells will arrive and settle in distant organs and only a small percentage of those will develop metastasis [43,44]. Even small tumors show dissemination. These CTC include mini-residuals and quiescent (sleeping) cells detached from the primary tumor [45]. The CTC colonize distant organ sites, where they are called DTC which may remain dormant or lead to metastatic disease. The appearance of CTC is therefore a mandatory step in establishing metastasis [46]. Usually, CTC present in the blood possess antigenic and/or genetic characteristics of the tumor type from which they arose [47]. Detection of CTC/DTC in blood or tissues has presented an opportunity to more fully understand the biology of metastasis, design new therapeutic strategies, and develop diagnostic and prognostic tools.

4. BREAST CANCER

Because mammaglobin A is highly specific for breast, most of the proposed uses for this marker are related to cancer involving this tissue.

Breast cancer is the most frequent cancer in women. It constitutes ~20% of all malignancy in women with a high and constantly increasing incidence. Currently, breast cancer affects ~6% of the female population [48].

Despite recent advances in early diagnostics and treatment strategies, breast cancer remains a leading cause of cancer-related death among women. Approximately 40% of patients eventually die from the disease. The development of metastases is the major cause of death [12].

Breast cancer dissemination should involve a succession of clinical and pathological stages starting with carcinoma *in situ*, progressing into invasive lesion and culminating in metastatic disease. Previously, it was thought that metastasizing breast cancer cells first colonized the lymph nodes before reaching peripheral blood and distant locations. It has now become clear that metastatic spreading occurs in ~50% of cases with apparently localized breast cancer (with negative nodal metastasis). In addition, clinically detectable metastases have only been reported in 5% of patients at the time of primary diagnosis, but up to 30% of patients with lymph node-negative disease will develop distant metastases within 5 years, probably succumbing to the disease [39,49–51].

Therefore, even early diagnosed patients who have undergone potentially curative surgery may suffer from recurrence as a consequence of undetected metastasis that occurred before treatment [43]. As such, it is important to detect occult metastasis in early-stage patients to provide better prognosis and potentially new treatment options. CTC already appear in the early stages of mammary carcinoma before development of metastasis and can be detected at the time of diagnosis in some patients [51,52].

5. DISSEMINATION SITES AND MATERIAL SOURCE FOR CANCER CELL DETECTION

In general, breast cancer is not curable once metastases are detected by "classical" means. These include clinical manifestations of spread, imaging methods (i.e., tomography), and serum marker assays, that is, cancer antigen 15.3 (CA-15.3) or carcinoembryonic antigen (CEA) [12].

To reveal the importance of DTC or CTC in the metastatic process, several immunologic-based analytical methods have been introduced into laboratory practice. Because of their rarity, detection of DTC or CTC requires analytical methods with extremely high sensitivity and specificity. In addition, this method should be highly reproducible and relatively easy to perform [53].

Although lymph nodes have been proposed as a potential starting material for detecting CTC and DTC, a significant number of lymph node-negative patients have developed metastatic disease. As such, the reliability of this approach has been questioned [12].

The bone marrow is a common site of settlement for DTC in breast cancer and other primary carcinomas even in the absence of lymph node metastasis or clinical signs of distant metastases [50]. Bone marrow aspiration is, however, invasive, time consuming, expensive, and painful. This approach is not amenable to multiple sampling particularly for monitoring treatment response [39].

Conversely, CTC detection in peripheral blood appears ideal. Sampling is relatively painless and can be performed repeatedly, allowing real-time monitoring of metastatic progression. In addition, many patients without DTC in the bone marrow present these cells in peripheral blood [12]. Several studies have demonstrated the presence of CTC in peripheral blood of patients with early-stage cancer without metastasis [54–56]. Sampling of CTC in cancer patients has been termed a "liquid biopsy" [57].

6. METHODS FOR CTC ANALYSIS

As mentioned above, technical challenges for CTC detection are related to their extremely rarity in peripheral blood and our ability to correctly identify these cells as tumor based. Human blood has large cellular content including white blood cells ($5–10 \times 10^6$/mL), red blood cells ($5–9 \times 10^9$/mL), and platelets ($2.5–4 \times 10^8$/mL). In contrast, very few CTC will be present even in known metastatic disease. It is approximated that there is less than one CTC per 10^5–10^7 mononuclear cells or per mL of blood [58]. It has been estimated that 1×10^6 tumor cells per gram of tumor tissue enter the bloodstream daily [53]. Shedding is discontinuous and CTC are heterogeneous. CTC colonization of distant organs is extremely inefficient. Most CTC are destroyed in the circulation or become dormant at distant sites due to the absence of a proper growth environment. However,

once metastases are established, subsequent spreading may become much more efficient and deadly [46].

The rarity of CTC has led to the development of specific methods for enriching these cells up to 10,000-fold [12].

6.1. Cell enrichment methods

CTC enrichment methods can be based on their physical (size, density) or immunologic characteristics (Fig. 6.3).

In the former, the most widely used approach has involved density gradient centrifugation in Percoll or Ficoll to separate the mononuclear cells, a fraction which typically contains the highest percentage of CTC [59]. A variation of this method is using OncoQuick® (Greiner Bio-One, Frickenhausen, Germany), a density gradient-based method that adds a porous membrane to prevent contamination of the mononuclear fraction with blood cells with higher buoyant density [46]. This approach has been shown to reduce the number of monocytes, lymphocytes, and platelets without compromising CTC recovery rate. A method based on CTC physical characteristics is size filtration. This method relies on the small diameter of most blood cells (8–11 μm) and the assumption that tumor cells are relatively larger (~30 μm) in breast cancer. Using this approach, most peripheral blood leukocytes can be removed by filtration through a polycarbonate 8-mm pore membrane [46]. The first filter-based method described was ISET® (Isolation by Size of Epithelial Tumor cells) (Rarecells, Paris, France) which separated fixed tumor cells using a disposable block containing the pored membrane in an automated filtration device. Enriched CTC were

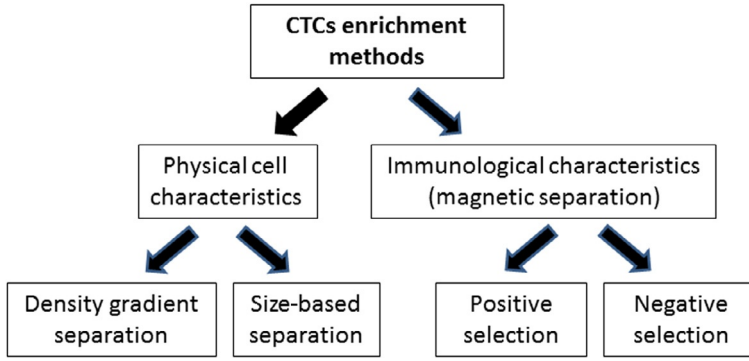

Figure 6.3 Types of methods used to recover circulating tumor cells.

identified by staining or immunocytochemistry [53]. The ScreenCell® filtration device (ScreenCell, Paris, France) is a recent advance that retrieves live CTC for cell culture [58]. CTC isolated by this method can be also be used for traditional staining, cell counting, immunolabeling, and molecular studies.

Other enrichment methods rely on the differential expression of specific antigens on the surface of CTC or mononuclear cells. Magnetic separation involves either positive selection via direct CTC capture or a negative selection by hematopoietic cell depletion. The antibodies employed in the positive selection methods target the epithelial tumor cell surface markers, while those used in negative selection assays are directed against the surface markers abundantly expressed in hematopoietic cells of different lineages. Currently, there are several commercially available CTC tests based on immunomagnetic separation. The magnetic activated cell sorting system MACS® (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) uses ferromagnetic microbeads coupled to an epithelial cell adhesion molecule (EpCAM) antibody. Following microbead incubation, epithelial tumor cells expressing EpCAM are chromatographically isolated in the presence of a strong magnetic field. Unlabeled cells are not captured, whereas tumor cells are trapped and subsequently recovered by removing the magnetic field. A similar methodology involves the use of Dynabeads® (Invitrogen, Carlsbad, CA) which also depends on the attraction by a magnetic field of cells recognized by antibodies coupled to ferromagnetic beads but does not require a separation column [46]. Another new automated immunomagnetic separation technology, the MagSweeper, also enriches circulating epithelial cells expressing EpCAM [57]. These strategies, however, are limited by CTC with absent or low target expression [46,60]. Enrichment techniques allow the identification of CTC at frequencies of one per 10^6–10^7 nucleated blood cells.

6.2. Detection methods for CTC

After successfully isolation, the second challenge is to correctly identify the tumor cells from other hematopoietic cells. False positives could confound clinical decision making resulting in inappropriate treatment choices and negative impact on quality of life and/or life expectancy [58]. The two main approaches for CTC detection are immunologic (monoclonal antibodies) and molecular [polymerase chain reaction (PCR)-based] assays that exploit tissue- and tumor-specific antigens (Fig. 6.4).

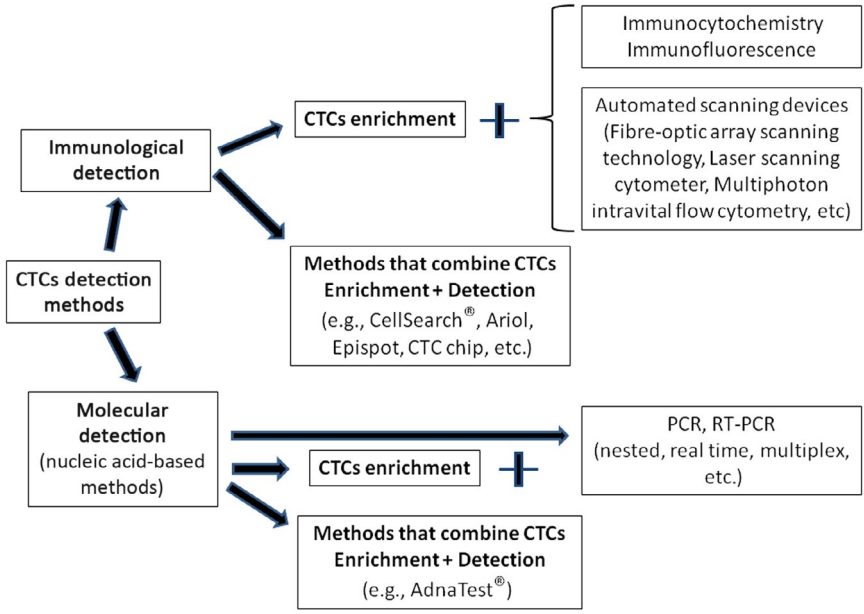

Figure 6.4 Classification of methods for circulating tumor cells detection.

Immunologic detection employs specific antibodies against antigens exclusively expressed by CTC versus other circulating nontumor or blood cells. Detection of CTC by trained Pathologists using immunocytochemistry is labor intensive and time consuming. This approach may take hours, perhaps days, if many samples require analysis [60]. Several fluorescence-based technologies are being used to improve immunocytochemical detection [61]. The development of automated scanning devices (Automated Cellular Imaging System, Chromavision®) has facilitated CTC detection and decreased interobserver variability. Examples of these devices include fiber-optic array scanning technology, laser scanning cytometer, and multiphoton intravital flow cytometry (for reviews, see references [46,61,62]).

Methods that combine immunomagnetic enrichment and detection have been developed to improve CTC isolation and detection. Examples are CellSearch®, Ariol and Epispot systems, and microfluidic devices such as the CTC chip (for reviews, see references [46,61–63]). Of these, CellSearch® (Veridex, Raritan, USA) is the only US Food and Drug Administration (FDA)-approved system for detection of CTC in patients with

metastatic breast, prostate, and colorectal cancer. This approach combines positive selection of cells with epithelial markers and negative selection of leukocytes. In this technique, CTC are magnetically captured, labeled with fluorescent antibodies, and detected with a semiautomated fluorescence microscope [63].

Using molecular methods, circulating nucleic acids and tumor cells have been detected using PCR which can detect specific DNA regions and reverse transcription PCR (RT-PCR), and variations thereof, which can detect mRNA expression of specific genes [39].

PCR has been used to identify and characterize CTC by genetic (allele-specific expression, microsatellite instability, loss of heterozygosity) and epigenetic (methylation status) changes specifically associated with cancer [12]. These include identification of tumor-associated point mutations in oncogenes or tumor suppressor genes. The latter approach, however, is complicated by poor specificity and the substantial genetic variability between tumors. In addition, mRNA is much less stable in plasma when compared to DNA. Although free plasma DNA could derive from CTC, primary or metastatic tumors as well as normal tissue [44], enrichment methods have the potential to eliminate most of these contaminants.

RT-PCR technique is the most frequently used method for molecular CTC detection because of its ability to analyze the expression of epithelial-specific or organ-specific marker signatures. Its sensitivity allows the detection of small amounts of specific mRNA species in the background of high total mRNA [63]. This approach is based on the fact that malignant cells often continue to express markers characteristic of or specific to its tissue of origin or with which the tumor shares histotype. These tissue-specific mRNA are highly suggestive of tumor spread when present at sites in which these transcripts are not normally present [64]. Because circulating free mRNA is highly susceptible to degradation by endogenous RNases, detection of tumor-specific mRNA could indicate the presence of viable tumor cells [39]. RT-PCR identifies CTC at the mRNA rather than protein level. This method does not require manual evaluation by highly trained personnel and facilitates the search for target genes relevant to metastasis [63].

RT-PCR detects one malignant cell out of 1–10 million normal cells (i.e., approximately one malignant cell in 1–10 mL blood). As can be expected, the sensitivity of this molecular approach is remarkably higher than traditional immunologic protein-based methods [46,65].

Paradoxically, RT-PCR is limited by its extremely high sensitivity. Since this technology is based on DNA amplification, even minimal

contamination could generate false positive results [43]. Although RT-nested PCR and quantitative real-time RT-PCR can increase sensitivity, these techniques also increase contamination risk [63]. However, the classical two-tube nested PCR protocol can be modified to reduce this potential complication [66]. A one-tube nested RT-PCR protocol, developed to detect mammaglobin A expression in blood samples and breast tissue, found no PCR product contamination [66].

Unfortunately, RT-PCR does not accurately quantitate CTC. Different transcripts can be expressed at various levels from different cells, that is, only the number of target transcripts can be estimated [61]. In addition, the detection of CTC by this approach requires the preparation of lysates that recover total RNA, thereby eliminating morphologic analysis [58]. Also, PCR sensitivity is likely influenced by markers not be equally expressed by all CTC due to heterogeneity, the presence of inhibitors in tissues and body fluids, and therapies that decrease target gene expression [46]. Although detecting several markers by multiplex RT-PCR may significantly improve sensitivity, this tactic has its own limitations including additional expense [61,63]. The only validated multimarker assay for CTC is AdnaTest® (AdnaGen AG, Langenhagen, Germany). This molecular profile for breast cancer includes three tumor-associated antigens and one control gene. The assay uses immunomagnetic enrichment with magnetic beads conjugated with both epithelial and tumor-specific antibodies [67]. Many CTC are under epithelial–mesenchymal transition and circulate with mesenchymal markers [68,69]. As such, markers for detection of mesenchymal-like subpopulations of CTC should be included. Unfortunately, the AdnaTest® does not detect mesenchymal-like CTC [70].

7. CLINICAL RELEVANCE OF CTC DETECTION

CTC are now emerging as relevant tumor biomarkers for diagnosis and therapeutic monitoring with the promise of more personalized medicine [61]. A more comprehensive understanding of biochemical and molecular characteristics of CTC is vital to investigating tumor biology, tumor cell dissemination, the biology of metastasis, and resistance to therapy [61]. Identification of CTC in patients with early-stage cancer may influence treatment modalities and reduce mortality [53,71]. Following surgical removal of the primary tumor, the presence of CTC could predict relapse and precede the increase in traditional serum markers [46]. In fact, several clinical

trials have investigated the value of CTC monitoring with therapeutic response and survival [54,72–75].

Although it has been suggested that peripheral blood CTC can be an independent prognostic factor in breast cancer [50,52,55,56,76,77], a consensus remains that more clinical evidence is needed prior to incorporating this approach in routine breast carcinoma studies [47,78]. The American Association for Cancer Research–FDA–National Cancer Institute "Cancer Biomarkers Collaborative" recently published a report on the validation and/or standardization of new biomarkers [79]. These recommendations aim to conclusively demonstrate applicability as well as accelerate development with respect to personalized medicine. This initiative also applies to CTC standardization with respect to specimen collection and processing, enrichment methods, analytical considerations, reporting format, quality control, and quality assurance [61].

8. MARKERS USED FOR MOLECULAR DETECTION OF CTC

An ideal molecular marker for CTC should be specific to tumor cells, not normal cells or to the tissue from which the tumor originated. The marker should also be easily detectable with little variance and bear clinical relevance. Despite this simple approach, there are few molecules that can satisfy these requirements [45].

Breast cancer cells commonly express several markers including cytokeratin (CK), CEA, Mucin 1, epidermal growth factor receptor (EGFR), EpCAM, human epidermal growth factor receptor 2, and others [12,80]. Unfortunately, these markers lack sufficient sensitivity and specificity for detecting breast carcinoma CTC [29]. Low specificity results from the presence of pseudogenes (genes that lack intronic sequences), illegitimate transcription in other cells and tissues (low-level ubiquitous transcription of a tissue-specific gene in non-specific cells), or induction of genes by cytokines and growth factors in hematologic disorders [12,81,82]. These factors may lead to false-positive PCR amplification indistinguishable from PCR products derived from intact mRNA [46]. Most molecular targets in these assays lack specificity, except for mammaglobin A in breast cancer [60].

9. MAMMAGLOBIN A AND BREAST CANCER

Different studies have evaluated the specificity of mammaglobin A in breast cancer.

Corradini *et al.* [83] studied a number of tumor markers in breast cancer tissue, peripheral blood, stem cells, and bone marrow. Cytokeratin 19 and 20, EGFR, CA15.3, and CEA were detected in samples from healthy donors, whereas mammaglobin A and maspin were not. Mammaglobin A and maspin were the only markers specific for breast cancer expressed in 97% and 80% of the breast carcinoma, respectively.

O'Brien *et al.* [84] compared the expression of three members of the human secretoglobins family (mammaglobin A, mammaglobin B, and lipophilin B) in nonmalignant breast tissues, fibroadenomas, and mammary carcinomas. Among these, only mammaglobin A was shown to be most specific for breast cancer.

A recent study examined the expression profiles of several molecular markers to evaluate the most suitable to identify primary sites in patients with metastatic carcinomas of different organs [85]. Mammaglobin A and prostate-derived Ets transcription factor were the only ones that detected breast cancer metastasis with high specificity.

9.1. Mammaglobin A as a CTC marker

Several reports confirmed the absence of mammaglobin A mRNA in blood samples from healthy donors [2,23,66,83,84,86–88]. Previous studies also described the absence of mammaglobin A expression in blood samples from benign breast disease [89,90].

Detection frequency of mammaglobin A expression in blood varies from 10% [86,88,89] to >35% [81,91,92] in breast cancer. Such discrepancies may be explained by differences in patient populations, methods of CTC enrichment, and mammaglobin A analysis.

Mammaglobin A expression evaluated by RT-PCR has become one of the main methods for CTC detection. It is the most studied breast cancer marker after cytokeratin 19 [12].

Zach *et al.* [81] evaluated CTC mammaglobin A expression in the peripheral blood of patients with breast cancer. This study found that this marker was not detected in healthy donors, whereas 25% of patients with breast cancer were positive for mammaglobin A mRNA. Similar findings were reported by Cerveira *et al.* [91] who detected mammaglobin A in 41% of patients with breast cancer but not healthy donors. In our laboratory, mammaglobin A expression was detected in 38% of blood samples from breast cancer patients, while all samples from healthy donors were negative [66]. Other authors also reported the specificity of this marker [93,94].

Grünewald *et al.* [95] reported that mammaglobin A showed higher specificity than EGFR and cytokeratin 19 (CQ-19) for detecting CTC in blood from breast cancer patients. No healthy individuals or patients with hematologic diseases were positive for mammaglobin A. However, 39% of healthy individuals were CQ-19 positive and 25% and 10% of patients with hematologic malignancies expressed EGFR and CQ-19, respectively.

Using nested RT-PCR, mammaglobin A and maspin expression were evaluated in blood and bone marrow samples exposed to cytokines [88]. Unlike maspin, mammaglobin A gene was never induced by any cytokine. Subsequent testing of maspin and mammaglobin A expression in leukocyte samples from patients with hematologic/inflammatory disorders indicated that maspin mRNA was expressed in 40% of these samples, whereas mammaglobin A was never detected. These results suggested that mammaglobin A was more specific than maspin and should be considered a reliable epithelial marker for CTC. Cytokine interference should be considered when interpreting molecular assays for detection of isolated tumor cells, that is, false positive results [88]. Because mammaglobin A is an epithelial marker, epithelial–mesenchymal transition CTC would not be detected, that is, false negative result.

A recent study compared three techniques for detecting CTC in blood samples from patients with metastatic breast cancer and healthy controls [96]. The techniques were CellSearch, AdnaTest Breast Cancer Select/Detect, and a previously developed real-time qRT-PCR assay for CK-19 and mammaglobin A transcripts. The qRT-PCR approach was found to be the most sensitive technique.

10. DETECTION OF MICROMETASTASIS

The detection of metastases in the axillary lymph nodes of breast cancer patients is the most powerful prognostic evidence in breast cancer and greatly influences disease management. Although the procedure initially involved full axillary lymph node dissection, sentinel lymph node biopsy was shown to accurately predict the involvement of the remaining nodes in the axilla and presented certain clinical advantages for the patient [97,98]. Sentinel lymph nodes will be the first to receive metastatic tumor cells and thus, most likely to contain them [99]. If the sentinel lymph nodes are determined to be free of disease, it is assumed that all other axillary lymph nodes will be negative and axillary dissection avoided. Today, sentinel lymph node biopsy is commonly performed to aid in the clinical staging

of patients with breast cancer and axillary lymph node dissection is performed only when the sentinel lymph node biopsy is positive [100,101].

Sentinel lymph nodes are typically assessed with hematoxylin and eosin (H&E) staining and occasionally by immunohistochemistry for some breast cancer markers [102]. Immunologic detection of mammaglobin A is also used for the identification of metastasis and occult micrometastases of breast cancer. It has been reported an improved sensitivity for the immunologic detection of breast cancer cells with an anti-mammaglobin A antibody versus EpCAM (BRST-1) and gross cystic disease fluid protein 15 (GCDFP-15) in a series of 70 breast cancer cases [103]. Other study compared the sensitivity of a mammaglobin A antibody cocktail with that of anti-GCDFP-15 for the identification of breast carcinomas [104]. The authors reported that the mammaglobin A immunostaining sensitivity for breast carcinoma was 93.1% whereas GCDFP-15 was 84.5%. In another study, the sensitivity and specificity of immunologic staining for mammaglobin A in metastatic breast carcinoma was 76% and 90%, respectively [105].

Because mammaglobin A is highly specific for mammary tissue, detection of the protein by immunohistochemistry is helpful in the diagnosis of lymph node metastases in breast cancer. The presence of mammaglobin A-positive cells in distant metastasis with an unknown primary tumor would suggest breast origin [106].

Recently, Tafreshi *et al.* [107] applied a high-resolution *in vivo* fluorescence imaging to detect lymph nodes metastasis of human breast cancer cells in a mouse model. This procedure could eliminate the need for surgical examination of patients to rule out nodal involvement. In this study, a monoclonal antibody specific for mammaglobin A was conjugated to a near-infrared fluorescent dye and delivered to the lymphatic system by peritumoral injection into the mammary fat pad of nude mice. Fluorescent imaging of mammaglobin A expressing cells that have spread to the axillary lymph nodes could then be performed. This novel approach offers a powerful targeted tool for *in vivo* studies of tumor cells within the lymphatic system, detection of tumor cells in lymph nodes, and may be applicable to monitoring antitumor therapy [107].

Detection of minimal breast cancer mRNA markers not expressed in lymphoid cells can be used to investigate node micrometastasis. Because mammaglobin A mRNA is highly specific, it can be used for detection of micrometastasis in sentinel lymph nodes to distinguish breast cells from lymphoid cells [14,108]. Commercial kits (Gene-Search Breast Lymph Node Assay; Veridex LLC, Raritan, NJ, USA) and instruments for a real-time

RT-PCR are available for detection of metastases in sentinel lymph nodes of patients with breast cancer. The assay assesses the expression of mammaglobin A and cytokeratin 19 in freshly homogenized nodal tissue [109]. During surgery, the assay could detect metastasis >0.2 mm in nodal tissue thus enabling a decision with respect to removal of additional axillary lymph nodes [110].

11. MAMMAGLOBIN A DETECTION: CLINICAL VALUE

Several studies have investigated the relationship of mammaglobin A in peripheral blood with breast cancer and other prognostic factors.

Some authors have reported a significant association, whereas others have described only a trend between mammaglobin A in blood and the absence of ER in breast cancer [66,94,111].

Recently, Ferro *et al.* [89] also found a clinically relevant association between mammaglobin A detection and ER-negative tumors. In our laboratory, the results of the odds ratio estimated that the chance to detect mammaglobin A in blood samples in the absence of tumor ER was more than 20 times higher than in their presence [66]. The presence of sex steroid receptors in the tumor further increased the likelihood of response to anti-hormonal treatment whereas their absence predicted disease relapse. As such, peripheral blood mammaglobin A could be associated with a higher risk of relapse.

On the other hand, most studies did not show a significant association between the detection of mammaglobin A and other prognostic factors including lymph node metastasis, tumor size, and histologic grade [29,66,92–94]. These findings suggested that mammaglobin A in blood could be an independent prognostic marker for breast cancer.

Approximately 30% of breast cancer patients without detectable lymph node disease at time of diagnosis can develop distant metastases and relapse after treatment. As such, monitoring CTC via mammaglobin A could assess disease progression as well as to identify those at increased risk of recurrence or more likely to develop a secondary tumor despite treatment. These patients could definitely benefit from closer follow-up and further adjuvant therapy if warranted.

Despite promising preliminary data, new and more comprehensive trials are clearly required to verify the expression mammaglobin A in peripheral blood of patients with breast carcinoma especially those without evidence of metastases.

12. MAMMAGLOBIN A AS A TARGET FOR CANCER THERAPY

Successful cancer-specific immunotherapy is based on the identification of tumor-specific antigens and their epitopes for both CD4 and CD8 T cells. Due to its high specificity, mammaglobin A is an attractive target for breast cancer therapy [22,112]. In fact, some studies have investigated this antigen for treatment and prevention of breast cancer [113–116]. Cytotoxic activity was evaluated against mammaglobin-A-positive breast cancer cell lines. In other studies, transgenic severe combined immunodeficient beige mice with actively growing tumors expressing mammaglobin A were vaccinated against the protein [117,118]. The immune response resulted in a statistically significant tumor regression suggesting therapeutic potential. Based on these results, a phase I clinical trial of a mammaglobin A cDNA vaccination in breast cancer patients with stage IV metastatic disease was conducted [119,120]. Although preliminary results indicated that the treatment elicited strong CD4 cellular effector immune response with antigen-specific cytotoxicity *in vitro*, this finding remains to be confirmed *in vivo*.

13. CONCLUSION

Mammaglobin A has become one of the most specific markers for breast cancer and for the detection of CTC and micrometastasis. To increase specificity, mammaglobin A has been combined with a number of other breast cancer markers in both experimental and commercial applications. Evidence from a number of studies suggests an independent prognostic value of mammaglobin A in blood from breast cancer patients and a potential application in therapeutic regimens. Despite the early and promising findings, it is clear that more comprehensive and well-controlled studies are warranted to more fully elucidate the role of this potential marker in breast cancer diagnostics.

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