Cancer-Specific mRNAs in Thyroid Carcinomas: Detection, Use, and Their Implication in Thyroid Carcinogenesis

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Introduction

MODERN advances in molecular technology have given us the chance to establish new strategies to diagnose and treat cancer [1, 2]. In order to implement such molecular-based technology, it is crucial to identify the distinct difference between benign and malignant cells. Without such identification, we can hardly tell which cells to detect or attack during the clinical course, because malignant cells usually exist surrounded by normal cells. Cancer cells are believed to be derived from normal cells via multiple damages to their genome, especially in oncogenes or anti- oncogenes that accelerate proliferation or foster malignant phenotypes such as the ability to invade the surrounding tissue or metastasize to distant organs. In recent decades, there have been extensive efforts to estimate the grade of malignancy of tumor cells by detecting their genomic changes. Efficient differentiation of malignant cells from benign, however, is not possible through simple analysis of their genomic alternations.

Ribonucleic acid (RNA) was at one time considered a rather difficult molecule to deal with, since it would easily be degraded by ubiquitously present RNase. In fact, the conventional methods of RNA analysis, e.g., Northern blot, differential hybridization, or in situ hybridization, are time- or material-consuming, hence technically quite demanding, and even up to several years ago, most RNA analyses involved only a few genes and a limited number of clinical samples. In recent years, however, remarkable advances have been made in the technologies of RNA analysis. In particular, the analyses to detect differentially expressed mRNAs in cancer tissues have become much easier to perform, leading to the discovery of many cancer-specific mRNAs for each malignancy. In this review, we summarize the recent advances in the study of cancer-specific mRNAs in thyroid carcinomas from two viewpoints: (1) how to detect them using clinical materials, and (2) how to implement them in molecular-based diagnosis. We then present our new hypothesis on thyroid carcinogenesis, which we feel more adequately explains the existence of cancer-specific mRNAs in thyroid carcinomas than the conventional multi-step carcinogenesis hypothesis.

Identification of cancer-specific mRNAs

In the 1980s, several oncogenes, such as c-MYC and c-FOS [3, 4], were reported to be overexpressed in thyroid carcinomas. These genes are related to the cell cycle and correspond to an increased rate of proliferation of cancer cells. This means that they are also expressed in normal thyroid follicular cells or in benign tissues and cannot be used in the molecular-based diagnosis of malignancies. Further, because most previous studies tended to examine only a limited number of cancer tissues, numerous reports have given discrepant results. On the other hand, NM23, which was regarded as a tumor-suppressor gene, was shown to be somewhat overexpressed in poorly differentiated carcinomas [5].

Before the development of polymerase chain reaction (PCR)-based technologies [6], it was fairly difficult to identify mRNAs expressed exclusively in malignant tissues. Nonetheless, increased expression of several genes, such as c-MET and CD26 in thyroid carcinomas, were found largely by chance [7, 8]. These genes were shown to be overexpressed especial-
ly in papillary carcinomas.

Reverse transcription (RT)-PCR, which requires only a small quantity of RNA, has facilitated RNA analysis using clinical samples. Since 1996, an increasing number of cancer-specific mRNAs have been found. Most of these mRNAs, e.g., cathepsin B, galectin-1 and c-erbB-2, are reported to be overexpressed in papillary carcinomas, while some genes are overexpressed in anaplastic carcinomas, and others in a part of follicular carcinomas (Table 1) [9–20].

Differential display (DD), a technique first developed by Liang and Pardee and currently used in some thyroid researches, completely changed the approach to differential screening of cancer-specific mRNAs [21, 22]. DD utilizes a 10 mer primer of random sequence as the 5’ primer for the PCR reaction. Differentially expressed mRNAs are rapidly screened and cloned by cutting out the bands of interest from the finger printing pattern of gel electrophoresis. However, the application of DD screening for the cancer tissues is quite difficult for the following two reasons. First, the high rate of false positive results makes the second screening difficult. Second, because DD amplifies relatively short PCR fragments in the 3’ untranslating region (UTR), individual differences in the 3’ UTR can be an obstacle in screening and can result in false positive results. Takano et al. developed a modified method of DD, Sequence Specific-Differential Display (SS-DD), for use in differential screening of cancer tissues (Fig. 1) [23]. SS-DD utilizes a 16 mer degenerate primer as the 5’ primer, and the PCR reaction is performed with a high annealing temperature, which prevents false positive bands in the display and makes possible the rapid screening of mRNAs in tissue samples. Using SS-DD, a fetal protein, oncofetal fibronectin (onfFN) mRNA, was found to be expressed in a restricted manner in papillary and anaplastic carcinomas and this fact was confirmed by immunohistochemistry and in situ hybridization [24–26]. Further, by SS-DD, decreased expression of c-FOS and acid ceramidase mRNAs has been reported in papillary carcinomas [27, 28].

By relying on 14–15 base cDNA sequences for gene identification, serial analysis of gene expression (SAGE) can generate a quantitative transcript profile easily, a task currently not possible using alternative transcript imaging technologies (Fig. 2) [29]. Since its introduction in 1995, SAGE has been used to analyze cDNA libraries derived from several carcinomas, and its reliability has been established. Recent studies show the establishment of gene expression profiles of normal thyroids and thyroid tumors and some specific changes in mRNAs in anaplastic carcinomas have been reported [30–32]. The use of this technique to provide gene expression profiles in normal thyroid and thyroid tumors may lead to an enhanced understanding of thyroid cell function and carcino-

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Tumor Type</th>
<th>Reference</th>
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<tr>
<td>MUC1</td>
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<td>k alpha-1 tubulin</td>
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Applications of cancer-specific mRNAs

When the expression of cancer-specific mRNAs clearly differs in benign and malignant tissues, they can be used for pre- or postoperative diagnosis of thyroid carcinoma. Thyroid tumors are often diagnosed by fine needle aspiration biopsy (FNAB) as well as by ultrasonography. Cytological examination of FNAB by a skillful pathologist who is an expert in thyroid tumors provides the most reliable information for the diagnosis of thyroid neoplasms [34]. In some clinical situations, however, slide samples are not adequate for cytological examinations due to poor fixation, and a well-trained expert pathologist is not always available for diagnosis. In such cases, a more objective method is required for exact diagnosis.

In 1996, Weiss et al. first applied a molecular-based technique for preoperative diagnosis of thyroid tumors by detecting a splice variant of MUC1 mRNA that is expressed in a restricted fashion in papillary carcinomas using the whole samples of FNABs [35]. Similary, Chiappetta et al. established a method of preoperative molecular-based diagnosis by detecting HMGII(Y) mRNA [36]. In 1997, Takano et al. introduced a new method of preoperative molecular-based diagnosis of thyroid carcinomas, which they named Aspiration Biopsy-RT-PCR (ABRP) (Fig. 3). ABRP allows us to perform cytological and molecular-based diagnoses simultaneously by extracting RNA
Fig. 3. ABRP procedures.

Tumor cells are aspirated by FNAB using a syringe with a 22-gauge needle (A). After preparing a sample on a slide glass for cytological examination (B) the needle is dipped into a 1.5-mL tube containing denaturing solution (C). The denaturing solution is aspirated in and out of the tube three times to lyse the cells inside the needle, and then the tube is stored at 4°C.

ABRP thus provides both RNA information by RT-PCR and a cytological diagnosis without further invasion to the patient. onIFN mRNA is abundantly expressed only in papillary and anaplastic carcinomas. They examined the expression of onIFN mRNA in 177 FNABs of thyroid nodules that were suspected of malignancy. Thirty-five (94.6%) of 37 samples that were diagnosed as papillary or anaplastic carcinomas by cytological examination showed a positive result by ABRP detection of onIFN mRNA, whereas only 4 (3.7%) of 109 samples that were cytologically diagnosed negative for both carcinomas showed a positive result. Among all the cases, 50 patients underwent surgery and a histological diagnosis was consequently made. The sensitivity and specificity of this method were 96.9 and 100%, respectively [38]. Further, an automated system that measures the quantity of a small amount of mRNA by monitoring the amplification rate in each PCR cycle (real-time quantitative RT-PCR) can be used to measure the ratio of onIFN and thyroglobulin mRNA in FNABs to distinguish these two carcinomas from benign tumors [39]. ABRP has also been applied for preoperative diagnosis of medullary thyroid carcinomas through the detection of RET, CEA, and calcitonin mRNAs, and for diagnosis of thyroid malignant lymphomas through determination of the monoclonality of immunoglobulin heavy chain mRNA [40, 41]. These methods, in which tumors are diagnosed by analyzing RNAs in aspirates, are designated aspiration-biopsy RNA diagnosis (ABRD). They are the promising applications of gene technologies in molecular-based diagnosis of cancer.

At present, these molecular-based diagnoses are quite reliable for the preoperative diagnosis of papillary, anaplastic and medullary carcinomas, and a part of malignant lymphomas. However, it is still quite difficult to diagnose follicular carcinomas preoperatively. A recent study has shown the usefulness of immunohistochemical study of galectin-3 for diagnosis of thyroid follicular carcinoma [42]. However, in another study, no significant differences were observed between follicular carcinomas and adenomas by quantitative measurement of galectin-3 mRNA [43]. Zeiger et al. measured the expression levels of telomerase reverse transcriptase (hTERT) in follicular tumors and showed that this method was to some extent useful in diagnosing follicular carcinomas, although they also described interference due to the contamination by lymphocytes, which express a considerable copy number of hTERT mRNA [44]. Kroll et al. reported some promising data that PAX-8-PPARγ1 fusion mRNA and protein were detected in 60% of thyroid follicular carcinomas but not in follicular adenomas, papillary carcinomas, or multinodular hyperplasias. However, because their study employed only a small number of follicular carcinomas, additional examination is necessary [45]. In conclusion, more intensive efforts will be needed to find a target mRNA that can be used to distinguish follicular carcinomas from adenomas at the molecular level.

Use of thyroid-specific mRNAs in diagnosis of thyroid carcinoma

Differentiated thyroid carcinomas (DTCs) express some mRNAs that are expressed only in thyroid tissues, such as thyroglobulin and thyroid peroxidase (TPO) mRNA. These mRNAs are also used for molecular-based diagnosis of thyroid carcinomas. Arturi et al. reported a PCR-based technique for detecting thyroid cancer metastases in small nodes by the amplification of thyroid-specific transcripts, e.g., thyroid stimulating hormone (TSH) receptor and thyroglobulin [46]. Among cases in which an adequate sample was obtained at fine needle aspiration, they reported a 100% correspondence between the PCR-based and histopathological diagnosis. The
genetic analysis was shown to be more sensitive and accurate than either cytological analysis or thyroglobulin measurement in the aspirates, suggesting that this technique may be a useful tool for diagnosis and follow-up of DTC.

Recent reports have demonstrated that RT-PCR can be used to detect circulating cancer cells in the peripheral blood of patients with malignancies. A sensitive RT-PCR assay amplifying thyroid-specific mRNAs such as thyroglobulin or TPO may be utilized for the early detection of DTC recurrence and thus may have important therapeutic and prognostic implications. In fact, some reports have shown the clinical usefulness of RT-PCR detection of thyroglobulin, TPO, and RET/PTC in the follow-up of DTC [47, 48]. For example, thyroglobulin mRNA in peripheral blood became detectable earlier than serum thyroglobulin in a case of recurrent thyroid papillary carcinoma. Further, Ringel et al. have reported the clinical usefulness of real-time quantitative measurement of thyroglobulin mRNA in the peripheral blood of patients with DTC [49]. However, two recent studies have raised some questions in regard to this method. Bojunga et al. have found that thyroglobulin mRNA expression is not specific to thyroid tissues and that RT-PCR detection of thyroglobulin mRNA is not correlated with a diagnosis of thyroid cancer in patients [50]. Takano et al. measured the copy number of thyroglobulin mRNA in peripheral blood from patients after total thyroidectomy by real-time quantitative RT-PCR, and found that thyroglobulin mRNA were detectable in all patients with or without distant or local metastasis, and no statistical difference between the samples from patients with and those without distant metastasis was observed [51].

Probably, an intensive re-evaluation, including a determination of what percentage of thyroglobulin mRNA derives from thyroid follicular or cancer cells, will be needed with regard to this method before considering its clinical applications.

The expression mechanism of cancer-specific mRNAs

It remains to be clarified why cancer-specific mRNAs, especially those overexpressed in fetal tissues, can clearly distinguish benign tissues from carcinomas, while genomic alternations, such as mutations in the RAS or P53 gene, cannot. It is rather odd that genomic alteration does not correspond with the malignant features if, as generally believed, cancerous characteristics are obtained by multiple changes in oncopogens or anti-oncogenes [52].

Like other carcinomas, thyroid carcinomas are believed to be generated from normal thyroid follicular cells by multi-step carcinogenesis [53]. According to this hypothesis, anaplastic carcinomas are generated from both follicular and papillary carcinomas by genomic changes, such as mutations in P53. Follicular carcinomas are generated from follicular adenomas, while papillary carcinomas are derived from some unknown precursor cells that are generated from normal thyroid follicular cells (Fig. 4). This rather complicated but widely accepted classical model of thyroid carcinogenesis, however, does not explain some of the recent molecular findings in thyroid carcinomas.

For example, mutations in P53, which are most often observed in anaplastic carcinomas, have been
recognized to be closely related to the aggressive features of these carcinomas [54]. However, recent studies have revealed that no mutation in \textit{P53} was observed in a considerable percentage of anaplastic carcinomas; further, these mutations are also observed in other types of tumors, even follicular adenomas [55]. Thus, it seems to be clear that mutations in the \textit{P53} gene are not necessarily responsible for the aggressive features of anaplastic carcinomas.

The existence of common genomic changes between differentiated carcinomas and anaplastic carcinomas may offer a direct proof of the multi-step carcinogenesis hypothesis. Tallini et al. examined the rearrangement of the \textit{RET} gene in both anaplastic carcinomas and differentiated carcinomas, and found that the rearrangement in the \textit{RET} gene is limited in papillary carcinomas and never observed in anaplastic carcinomas [56]. Mutations in some genes, such as the \textit{RAS} gene, are frequently observed in both differentiated and undifferentiated thyroid carcinomas [57]. Despite concerted efforts to find a common mutation in such frequently mutated genes in anaplastic carcinomas and co-existing differentiated tumors, no such cases have been reported. So far, there is no direct evidence to prove the succession of genomic changes from differentiated carcinoma to anaplastic carcinomas, which raises a question in regard to the hypothesis that these aggressive carcinomas derive from normal thyroid follicular cells by the accumulation of genetic changes in their genome.

The expression of fetal proteins, such as onfFN, is usually limited to two carcinomas, papillary and anaplastic carcinomas. This fact suggests the biological similarity of these two carcinomas. These considerations lead to the key question, “Where do these two carcinomas derive from?” The fetal thyroid originates in the pharynx and gradually moves to the front of the neck as it slowly grows. This indicates that fetal thyroid cells have the ability to move through other cells, i.e., the ability to induce invasion or metastasis, and that they grow slowly, which are the same characteristics we observe in DTCs. Further, in the mouse thyroid, fetal thyroid cells express thyroglobulin before they form follicles [58]. Because onfFN mRNA is known to be expressed in a wide variety of fetal tissues, the fetal thyroid is likely to express onfFN mRNA [59]. These facts indicate that fetal thyroid cells have characteristics quite similar to those of thyroid papillary carcinoma cells. It is thus more reasonable to assume that thyroid carcinomas are generated directly from the remnants of fetal thyroid cells, rather than from rarely proliferating thyroid normal follicular cells by de-differentiation.

**The germ-cell carcinogenesis hypothesis**

Considering these facts, we propose a new model of thyroid carcinogenesis, which we term the “germ-cell carcinogenesis” model. In multi-step carcinogenesis, thyroid carcinomas are generated from normal thyroid follicular cells via multiple genomic changes that foster the development of their cancerous characteristics. In germ-cell carcinogenesis, on the other hand, thyroid carcinomas derive from the remnants of fetal thyroid germ cells (thyrobasts), which have the ability to invade and metastasize, unlike normal thyroid follicular cells (Fig. 5).

This hypothesis has two evident advantages over conventional multi-step carcinogenesis. First, micro papillary carcinomas, which grow quite slowly and are often observed in autopsies, show a distinct morphological difference from normal follicular cells [60]. It is hard to believe that these carcinoma cells

![Multi-step Carcinogenesis](image1)

**Fig. 5.** Comparison of multi-step carcinogenesis and germ-cell carcinogenesis.
obtain their cancerous characteristics via multi-step carcinogenesis, since they do not divide many times before they are recognized as carcinomas, and thus are not likely to have undergone a dramatic change. In germ-cell carcinogenesis, on the other hand, these cells are considered to be derived from germ cells, which already possess the cancerous characteristics. Thus, they already resemble cancerous cells before proliferation. Second, in germ-cell carcinogenesis, genomic changes in cancer cells do not play a major role in the expression of cancerous characteristics unless such changes prevent the germ cells from differentiation, whereas the expression of fetal protein mRNAs is considered to prove that these cells are remnants of fetal cells. Thus, germ-cell carcinogenesis, but not multi-step carcinogenesis, explains why cancer cells can be distinguished from benign ones by the expression of fetal protein mRNAs, and why they cannot be distinguished by their genomic changes.

Fig. 6 summarizes the concept of germ-cell carcinogenesis. Papillary carcinomas are derived from the remnants of thyroblasts, which express both onfFN and thyroglobulin mRNAs. Anaplastic carcinomas may derive from more poorly differentiated precursor cells, which express onfFN mRNA but not thyroglobulin mRNA. These cells already possess cancerous characteristics, and as they proliferate, secondary genomic changes occur. Germ-cell carcinogenesis regards carcinogenesis as an abnormal development of fetal thyroid cells, but not de-differentiation of normal or benign thyroid follicular cells.

There has been no conclusive evidence to prove either multi-step carcinogenesis or germ-cell carcinogenesis. However, two recent reports strongly support the germ-cell carcinogenesis hypothesis in thyroid carcinomas. First, in the Chernobyl accident, radioactive iodine, which does not have the ability to induce thyroid cancer in adults, induced papillary carcinomas in young children and infants [61]. Interestingly, although this happened in an iodide-deficient area, where follicular carcinomas usually make up a considerable percentage of thyroid malignancies, almost all radiation-induced tumors were papillary carcinomas. These facts strongly suggest that thyroid carcinomas, especially papillary carcinomas, are derived from some unknown source which exists only in young children or babies but not in adults, and in germ-cell carcinogenesis this is recognized as thyroblasts. Second, Jihaing et al. found that in RET/PTC1 transgenic mice develop thyroid papillary carcinoma and congenital hypothyroidism due to their lack of normal thyroid cells [61]. These authors attributed the observed hypothyroidism to

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**Fig. 6.** Germ-cell carcinogenesis of thyroid carcinoma.
the de-differentiating effect of the RET/PTC1 protein on thyroid follicular cells. These results can be more simply explained if we hypothesize that the transgene RET/PTC1 prevents the differentiation of thyroblasts into normal thyroid follicular cells. Thus the remnants of thyroblasts grew gradually until birth, then resulted in the development of papillary carcinomas and congenital hypothyroidism.

Cancer-specific mRNAs: a lesson from the recent studies

Recent data from the analyses of cancer-specific mRNAs in thyroid carcinomas have given us deeper knowledge and insight in regard to the biological features of thyroid tumors. The effectiveness of molecular-based techniques depends greatly on the basic model of carcinogenesis. Multi-step carcinogenesis theory has made a great contribution to the establishment of gene diagnosis of hereditary cancers. However, further efforts and fresh ideas would seem to be needed to overcome the difficulties in diagnosing sporadic thyroid carcinomas. Our new model of thyroid carcinogenesis, as described above, may help in the reconsideration of the features of thyroid carcinomas and their clinical management.

Summary

Molecular-based diagnosis of thyroid carcinomas can be more easily established by utilizing specific mRNAs that are expressed in a restricted manner in cancer tissues. Accordingly, several cancer-specific mRNAs in thyroid carcinomas have been identified by means of sequence specific-differential display (SS-DD), serial analysis of gene expression (SAGE) and other new techniques. By using these cancer-specific mRNAs, some new methods of preoperative diagnosis of thyroid carcinomas have been developed. In one such method, Aspiration Biopsy-Reverse Transcription-Polymerase Chain Reaction (ABRP), RNA is extracted from leftover cells within the needle used for fine needle aspiration biopsies (FNABs), thereby allowing cytological and molecular-based diagnoses to be performed simultaneously. ABRP provides both RNA information and a cytological diagnosis without further invasion to the patient. By ABRP detection of cancer-specific mRNAs, papillary, anaplastic and medullary carcinomas and a part of malignant lymphomas can be accurately diagnosed preoperatively. It remains to be clarified why cancer-specific mRNAs, especially those that are overexpressed in fetal tissues, can clearly distinguish benign tissues from carcinomas, while genomic alternations, such as mutations in the RAS or P53 gene cannot. Further, the widely accepted hypothesis of multi-step carcinogenesis cannot explain some of the clinical and experimental findings of thyroid carcinomas. Considering these facts, we propose a novel hypothesis of thyroid carcinogenesis, the “germ-cell carcinogenesis” hypothesis, in which cancer cells derive from the remnants of fetal thyroid germ cells (thyroblasts) instead of normal thyroid follicular cells.

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