Gene expression profiles in thyroid carcinomas

T Takano1, Y Hasegawa1, F Matsuzuka3, A Miyauchi3, H Yoshida3, T Higashiyama2, K Kuma3 and N Amino1

1Department of Laboratory Medicine, and 2 Surgical Oncology, Osaka University Medical School, D2, 2-2 Yamadaoka, Suita, Osaka 565-0871, 3Kuma Hospital, 8-2-35, Simoyamatedori, Chuo-ku, Kobe, Hyogo 650-0011, Japan

Summary The gene expression profiles of human thyroid carcinomas were analysed by serial analysis of gene expression (SAGE) which allows quantitative and simultaneous analysis of a large number of transcripts. More than 29 000 transcripts derived from a normal thyroid tissue and four thyroid tumours were analysed. While extensive similarity was noted between the expression profiles of the normal thyroid tissue and three differentiated thyroid tumours, many transcripts, such as osteonectin, a-tubulin, glyceraldehyde-3-phosphate dehydrogenase, glutathione peroxidase, and thyroglobulin, were expressed at extremely different levels in differentiated and undifferentiated carcinomas. These data provide new information that might be used to identify genes useful for the diagnosis and treatment of thyroid carcinomas. © 2000 Cancer Research Campaign

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Recent advances in molecular technology suggest the potential for more efficient and effective molecular-based diagnoses and therapies. Many studies, such as those concerning p53, RAS, RET, and thyrotropin receptor, have improved our understanding of thyroid carcinogenesis (Farid, 1996). However, more intensive studies to further clarify the molecular mechanism of carcinogenesis are necessary before we select the molecular targets for these technologies.

In the thyroid, as in other organs, genes that are found to be differentially expressed between normal thyroid tissue and thyroid carcinomas can be used as targets for molecular-based diagnosis and therapy (Chiappetta et al, 1998; Takano et al, 1998, 1999). Recent developments in technologies aimed at identifying differentially expressed genes, such as differential hybridization and differential display, have identified some genes the expression of which is restricted to thyroid carcinomas (Gonsky et al, 1997; Takano et al, 1997; de Nigris et al, 1998). However, the data made available by these methods are still insufficient for a comprehensive evaluation of all genes involved in carcinogenesis.

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 (Velculescu et al, 1995), and is less laborious than the body mapping method which can generate similar profiles (Matsubara and Okubo, 1993). Since its introduction in 1995, SAGE has been used to analyse cDNA libraries derived from several carcinomas and its reliability has been established (Zhang et al, 1997; Hibi et al, 1998). We describe here the use of SAGE to provide gene expression profiles in normal thyroid and thyroid tumours, a technique that may lead to an enhanced understanding of thyroid cell function and carcinogenesis.

MATERIALS AND METHODS

Materials

Tissue samples for SAGE were obtained surgically from a normal thyroid tissue adjacent to a follicular adenoma in a 43-year-old female, a follicular adenoma in a 43-year-old female, a papillary carcinoma in a 32-year-old female, a widely invasive follicular carcinoma in a 35-year-old female, and an anaplastic carcinoma in a 77-year-old female. Tissue samples from three normal thyroids, follicular adenomas, papillary carcinomas, follicular carcinomas and anaplastic carcinomas were also collected for reverse transcription-polymerase chain reaction (RT-PCR) analysis. Thyroid tumours were classified histopathologically according to the WHO histological classification of thyroid tumours (Hedinger et al, 1989). Total cellular RNA was extracted according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) and poly A RNA was purified with oligotex-dT30 (Takara, Shiga, Japan) according to the manufacturer’s protocol.

SAGE protocol

The SAGE method was performed as described previously with some modifications. 3 µg of poly A RNA was converted to double-stranded cDNA with a BRL synthesis kit (Gibco BRL, Tokyo, Japan) according to the manufacturer’s protocol except for the inclusion of primer biotin-5'-T3'-3'. The cDNA was cleaved with Nia III (anchoring enzyme) (Daiichi-Kagaku, Tokyo, Japan). After capture of the 3' cDNA fragments on streptavidin-coated magnetic
beads (Dynal, Tokyo, Japan), the bound cDNA was divided into two pools, and one of the following linkers containing a recognition site for BsmFl (Daichi-Kagaku) was ligated to each pool:

linker 1, 5′-TTTGGATTTCTGGTTCAAGCTACAGT-GCCTTAATAGGACATG-3′, 5′-TCCCTATTAAGGC-CATTGTGATACCTGCAGCAGAATTC (amino modification C7)-3′.

linker 2, 5′-TTTCTGCTGATTAATCTGTAATCC 5′-TCCCGCTACATCGTTAAGAAGCTTCTAATCC (amino modification C7)-3′.

Since BsmFl (tagging enzyme) cleaves 14 bp away from its recognition site, and the NlaIII site overlaps the BsmFl site by 1 bp, a 15 bp SAGE tag was released with BsmFl, SAGE tag overlaps were filled in with Klenow (Takara), and tags from the two pools were combined and ligated to each other. The ligation product was amplified by 15 cycles of PCR using 5′-GGATTTGCTGGTGCAGTACA-3′ and 5′-CTGCTCGAAT-TCGAAGCCTCTACTAG-3′ as primers. All the linkers and primers were obtained from Gibco BRL. The PCR products were analysed by polyacrylamide gel electrophoresis (PAGE), and the PCR product containing two tags ligated tail to tail (ditag) was excised. The PCR product was re-amplified by 20 cycles of PCR using the same primers, purified by PAGE, then cleaved with NlaIII. The band containing the ditags was excised and self-ligated, then cleaved with SphI (Takara). The concatenated products were separated by gel filtration using a Sephadex 400R (Amersham Pharmacia, Tokyo, Japan), then cloned into the SphI site of pGEM-SZI (+) (Promega, Tokyo, Japan). These procedures produced about 500 white colonies per reaction. Colonies were screened for inserts by PCR using primers which sequences located outside the cloning site. Colonies containing inserts of about 400 bp in length were selected for the further analysis. Plasmids from selected clones were purified by an automatic plasmid isolation system PI-100 (Kurabo, Osaka, Japan) then sequenced with Taq FS Dye Primer kits (PE Biosystems, Tokyo, Japan) and analysed using a 373 ABI automated sequencer (PE Biosystems), following the manufacturer’s protocol. Sequence files were analysed by the SAGE software and the tag sequences were analysed by the BLAST program of the DNA Data Bank of Japan (Mishima, Sizuka, Japan). The occurrence rates of tag sequences were calculated by dividing the number of occurrences of a particular tag sequence by the total tag count.

Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR analyses of 4 representative mRNA sequences were performed as previously described (Takano et al, 1997). The sequence of the 5′ primers are 5′-GGATTTGCTGGTGCAGTACA-3′ (base 1511–1530) (Swaroop et al, 1988) for

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Table 1 SAGE analysis of a normal thyroid and a follicular adenoma

| Normal thyroid | Follicular adenoma |
|---------------|--------------------|
| Count | Sequence | Definition | Count | Sequence | Definition |
| 64 | CCACGCCGACCT | EST A1081056 | 144 | CCGTGAAAAC | thyroglobulin |
| 63 | CGGGTAGAAA | thyroglobulin | 56 | CCGTGATACATC | 5′-nucleotidase |
| 55 | ACTTTCTGACCT | mitochondrial cytochrome oxidase subunit 1 | 54 | ACTTTTTCCTA | mitochondrial cytochrome oxidase subunit 1 |
| 50 | GTGAAACCCTC(A) | 1. Alu transcript | 54 | CCACGCAGCT | EST A081056 |
| 49 | CCGTGAAACCCT | 2. obese protein | 47 | CGGTGAAGCA | EST A081056 |
| 48 | GTGAAACCCTC(A) | 3. platelet-activating factor acetylhydrolase 2 | 38 | GTGAAACCCC | putative serine-threonine protein kinase |
| 33 | GTGAAACCCCT | putative serine-threonine protein kinase | 34 | GTGTTGAGAGA | elongation factor 1-alpha |
| 30 | TGTGTGAGA | elongation factor 1-alpha | 33 | GTGAAACCCCCT | 1. Alu transcript |

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Table 2  SAGE analysis of papillary, follicular and anaplastic carcinomas

| Papillary carcinoma | Follicular carcinoma | Anaplastic carcinoma |
|---------------------|----------------------|----------------------|
| **Total no. of tags = 6435, no. of unique tags = 662** | **Total no. of tags = 5275, no. of unique tags = 630** | **Total no. of tags = 7124, no. of unique tags = 849** |
| **Count** | **Sequence** | **Definition** | **Count** | **Sequence** | **Definition** | **Count** | **Sequence** | **Definition** |
| 159 | CCCATGGTCC | mitochondrial cytochrome oxidase subunit 2 | 188 | CGGTGAAAAA | thyroglobulin | 87 | ACTTTTCCCA | mitochondrial cytochrome oxidase subunit 1 |
| 146 | CACCTAATTG | mitochondrial ATP synthase 6 | 55 | CCGTGATCC | 5'-nucleotidase | 64 | CCCATGGTCC | mitochondrial cytochrome oxidase subunit 2 |
| 122 | ACCCTGGGCCC | mitochondrial NADH dehydrogenase 1 | 51 | CCCATGGTCC | mitochondrial cytochrome oxidase subunit 2 | 63 | GGGTGTTGTTA | beta 2 microglobulin |
| 93 | TGATTTTACT | mitochondrial cytochrome c oxidase subunit 3 | 45 | ACCCTGACCA | mitochondrial NADH dehydrogenase 3 | 60 | ATGGTAGAGAG | SPARC | |
| 84 | TTGGGTTTTC | fentin H chain | 44 | ACCCTAATCC | mitochondrial ATP synthase 6 | 59 | TTGATAGCCAC | mitochondrial NADH dehydrogenase 4 |
| 79 | GTGAAACCCCGG | | 38 | CCATCCGCTTCA | EST A0108156 | 55 | TGGAATGACG | alpha 1 collagen (polymorphic transcript) |
| 3. | | | 34 | GTGAAACCCCGG | 1. | | |
| 1. | | | | | | |
| 2. | | | | | | |
| 18 | CCCATGGTCC | mitochondrial cytochrome oxidase subunit 2 | 18 | CCCATGGTCC | mitochondrial cytochrome oxidase subunit 2 | 18 | CCCATGGTCC | mitochondrial cytochrome oxidase subunit 2 |
| 18 | CCCATGGTCC | mitochondrial cytochrome oxidase subunit 2 | 18 | CCCATGGTCC | mitochondrial cytochrome oxidase subunit 2 | 18 | CCCATGGTCC | mitochondrial cytochrome oxidase subunit 2 |
| 18 | CCCATGGTCC | mitochondrial cytochrome oxidase subunit 2 | 18 | CCCATGGTCC | mitochondrial cytochrome oxidase subunit 2 | 18 | CCCATGGTCC | mitochondrial cytochrome oxidase subunit 2 |
osteonectin, 5′-GGATTTGCTGGTGCAGTACA-3′ (base 1021–1040) for α-tubulin (Cowan et al, 1983), 5′-CCAAGGTCATCCATGACAAC (base 557–576) for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Arcari et al, 1984), and 5′-ACGTGTCCTACCTATGTGTC-3′ (base 981–1000) for glutathione peroxidase (Takahashi et al, 1990). A poly A-anchor primer DDR (5′-ATGCGAATTCGTTTTTTTTTTTTTTTTTT-3′) was used for the 3′ primer. RT was performed using 1 µg of total RNA in an RT mixture containing 40 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM MgCl2, 0.5 mM dNTPs, 200 U Moloney murine leukaemia virus reverse transcriptase (Gibco BRL), 2 U/µl RNase inhibitor (Takara), and 2.5 µM oligodeoxythymidylic acid (Gibco BRL) in a total volume of 20 µl at 37˚C for 60 min. For PCR, each reaction mixture consist of 1 µl of cDNA, 0.5 µM each primer, 2 µl of 10 × Ex Taq buffer (Takara), 1.6 µl of 2 mM dNTP mix (PE Biosystems) 0.5 U of Ex Taq polymerase (Takara), and nuclease-free water to a final volume of 20 µl. The reaction mixture was subjected to 25 cycles of denaturation (94˚C; 1 min), annealing (55˚C; 1 min), and extension (72˚C; 1 min). After PCR amplification, 5 µl of reaction mixture was run on 1.5% agarose gel. The gel was stained with SYBR Green I (Takara), then analysed with a Fluor Imager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

SAGE libraries were constructed from mRNAs isolated from a normal thyroid tissue sample and four thyroid tumours. In total, 29,275 tags were sequenced, representing about 600 unique tags in each tissue (Tables 1 and 2). The majority of the highly expressed sequences in each tissue code mitochondrial and ribosomal proteins. The tag sequence of thyroglobulin mRNA was highly expressed in the normal thyroid and the 3 differentiated thyroid tumours but not in the anaplastic carcinoma. In the 2 differentiated carcinomas, high expression levels of the tag sequence of cathepsin B were observed. In the anaplastic carcinoma, most of the highly occurring tag sequences were derived from housekeeping genes in addition to mitochondrial and ribosomal sequences. Some sequences that were only seldom observed in the differentiated carcinomas, such as those of osteonectin and collagen genes, were also highly expressed.

To generate a profile of the relative gene expression patterns in each tumour, the occurrences of each tag identified in the tumour library were compared with those observed in the libraries of the other tumours or of the normal thyroid. Representative sequences are listed in Tables 3 and 4. The tag sequences that code mitochondrial and ribosomal proteins were excluded from the lists. A small number of tag sequences showed extreme differences in the expression levels among the normal thyroid and differentiated tumours. In contrast, among the 97 tag sequences which occurred 10 times or more, 29 (29.8%) and 27 (27.8%) sequences occurred at rates 10-fold or more than those in papillary and follicular carcinomas, respectively, which indicates that the expression profile of the anaplastic carcinoma is much different from those of the differentiated carcinomas.

Expression levels of some genes whose tag sequences were differentially expressed in the anaplastic carcinoma were examined by semi-quantitative RT-PCR. Semi-quantitative RT-PCR
Table 3 List of differentially expressed genes in the normal thyroid (N), follicular adenoma (F), papillary carcinoma (PC), and follicular carcinoma (FC)

| Count | N | F | Sequence | Count | F | PC | Sequence | Count | F | FC | Sequence |
|-------|---|---|----------|-------|---|----|----------|-------|---|----|----------|
| 25    | 0 | TCAAGCCATC | 1     | 20  | GCAAGCCAAC | 0 | TGTGACGCCG |
| 18    | 0 | TTGGCTTGCT | 1     | 18  | ACACAAGCAAG |
| 14    | 1 | GAAATAAAGC | 0     | 13  | ACCCTGTGCC |
| 14    | 0 | CCCAACGGGC | 0     | 12  | L-iditol-2 dehydrogenase |
| 13    | 1 | AAGGGAGCAC | 0     | 12  | GGCATCCCT |
| 13    | 1 | GGATATTTGG | 0     | 11  | ATGGCTGGTA |
| n = 6 |   |          |       | 10  | TGTCCCCTGTG |

α-1 collagen type 1 transcription factor ETR103

α-1 collagen type 1

n: the number of sequences occurred at rates ten-fold or more than those in the compared tissue.
|        | Count | PC | FC | Sequence     |        | PC | AC | Sequence     |        | FC | AC | Sequence     |
|--------|-------|----|----|--------------|-------|----|----|--------------|-------|----|----|--------------|
| 0      | 26    | TGTGACGCCG | n: no match | 4     | 60  | ATGTGAAGAG | n: no match | 188   | 0    | CGGTGAAAAA |
| 11     | 0     | AAAACATTCT | EST AA095120 | 8     | 51  | GTTCACATTA  | EST AA533220 | 5     | 63  | TTGTGTCTAA |
| 10     | 0     | CTGACCTGTG | MH C HLA-B7 class I cell surface glycoprotein heavy chain | 0     | 37  | ACCAAAAACC  |        | 3     | 60  | ATGTGAAAGG |
|        | 0     | no match   | EST AA533220 | 0     | 35  | CGGTGAACCT  | 0     | 30    | 0    | CGGTGAAGCA |
| 2      | 29    | TACCATCAAT  |        | 0     | 26  | AGAAAAAAA   |        | 30    | 0    | CGGTGAAGCA |
| 1      | 28    | TTGACAGTTT  |        | 0     | 26  | AGAAAAAAA   |        | 29    | 0    | AOGGAGGCCC |
|        | 0     | no match   | EST AA533220 | 2     | 23  | GGGCATCTCT  | HLA-DR alpha-chain | 0     | 26  | TTGACAGTTT |
|        | 2     | 21          | TGTACCTCT  | 2     | 21  | TGTACCTCT   | alpha-tubulin   | 2     | 21  | TTGACAGTTT |
| 1      | 21    | CTTGAAATCC  | EST H87461 | 1     | 21  | TGTACCTCT   | collagen VI alpha-1 | 1     | 21  | TTGACACTT |

*n: the number of sequences occurred at rates ten-fold or more than those in the compared tissue.*
confirmed increased expression of osteonectin, α-tubulin, and GAPDH, and decreased expression of glutathione peroxidase mRNA in 3 anaplastic carcinomas (Figure 1).

DISCUSSION

In this study, we used SAGE to analyse cDNAs from tissues of a normal thyroid and 4 thyroid tumours and created expression profiles for each tissue. In our results, some tag sequences corresponded to more than one gene. It was not possible, by means of only SAGE-data analysis, to determine whether all of the corresponding genes were expressed in the tissue. In the case of these sequences, further analyses, such as Northern blot or quantitative RT-PCR analyses, may be needed. Some tag sequences with no homology to known genes appear on the list. These sequences might be derived from some unknown genes, although the possibility of interference by the individual variations in the 3′ untranslated region of mRNAs should be also considered.

Pauws et al recently described the application of SAGE to create an expression profile of the normal thyroid (Pauws et al, 2000). Their data are quite similar to ours in that the majority of the highly expressed sequences coded mitochondrial or ribosomal proteins and the thyroglobulin gene was highly expressed. However, while they detected 24 tags of thyroid peroxidase, we detected none in the normal thyroid tissue and only 2 in the follicular adenoma. The effects of some endemic factors, such as iodine uptake, may explain this discrepancy. Further, because we performed SAGE analysis on a smaller scale than they did, only about 600 unique genes were identified. Thus, the analyses were limited to abundantly expressed sequences, and this is another reason why most of the thyroid-specific genes with moderate or low expression levels could not be detected.

In our study, the tag sequences of some genes, such as thyroglobulin, cathepsin B, and thyminosin beta 10, were expressed in the benign and malignant tumours in a manner similar to that in previous reports (Brabant et al, 1991; Shuja and Murnane, 1996; Califano et al, 1998), suggesting the reliability of these SAGE data. For example, the tag sequence of cathepsin B occurred at a much higher rate in the papillary and follicular carcinomas than in the normal thyroid or the follicular adenoma.

In the anaplastic carcinomas, most of the highly occurring tag sequences code mitochondrial proteins, ribosomal proteins, or housekeeping genes, such as GAPDH. Interestingly, the products of some of these genes are already being used as serum tumour markers such as beta 2-microglobulin and ferritin. Thus, some of the genes identified here and shown to have high occurrence rates could be candidate markers of follicular carcinomas.

One of the most difficult distinctions in thyroid pathology is the differentiation between benign follicular adenomas and follicular carcinomas (Rosiś and Carcangiu, 1987). Preoperative differentiation of follicular adenomas and carcinomas by cytopathological examination is quite difficult; accordingly, there has been a concentrated effort to establish a definite molecular marker of follicular carcinoma. Although only several differentially expressed genes were identified in the present study, some of the genes with known and unknown properties as listed in Table 3 may be candidate markers of follicular carcinomas.

In conclusion, in the present report, we analysed the gene expression profiles in the normal thyroid and 4 representative thyroid neoplasms. The results of this study may provide clues toward not only the establishment of a molecular-based diagnosis and therapy, but also an improved understanding of thyroid function and tumorigenesis.

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