Detection of *erbB*-2 Amplifications in Tumors and Sera from Esophageal Carcinoma Patients[1]

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ABSTRACT

We used TaqMan PCR to detect quantitative anomalies of tumor markers in both tumor and serum DNA from esophageal cancer patients. We demonstrated the potential of this methodology by detecting *erbB*-2 amplifications in a plurality of esophageal tumor samples. These amplifications were corroborated by Southern blots. We then showed the potential of this methodology to detect quantitative anomalies of *erbB*-2 in serum DNA from individuals with a corresponding amplification in the tumor. The capability of TaqMan PCR to detect abnormalities in serum of esophageal cancer patients creates an opportunity to diagnose esophageal cancer and to monitor the outcome of treatment with a blood test.

INTRODUCTION

Recent studies indicate that soluble tumor DNA is found in the serum and plasma of cancer patients; alterations of both microsatellite and specific sites corresponding to the lesions in tumors have been identified in the serum or plasma of patients with several cancers (1–5). Microsatellite analysis of serum represents a noninvasive method for the detection of circulating tumor cell DNA. This encouraged us to determine whether such gene copy alterations could be detected in an analogous manner by another noninvasive molecular methodology, TaqMan PCR. As a model system, we followed the amplification status of *erbB*-2 in esophageal tumors.

The incidence of esophageal adenocarcinoma has increased markedly in the past two decades (6). The mortality associated with esophageal adenocarcinoma is high because many tumors are not detected until the disease has progressed to an advanced stage. Even when the primary tumor is resectable, the overall 5-year survival rate is still low (7). New approaches to accomplishing early detection and to monitoring the course of therapy would benefit the clinical management of this disease.

About 20% of esophageal adenocarcinomas show amplification of the *erbB*-2 oncogene (8).[3] Amplification of oncogenes in cancer can be detected by either quantitative Southern blotting (9), which requires large amounts of DNA, or by FISH[4] (10, 11), which requires intact cells. However, neither of these techniques can be used to monitor changes in the small amounts of acellular DNA present in serum. Deletion of tumor suppressor genes in cancer can be detected by LOH analysis (12), but it requires polymorphic markers. Recently, we developed a fluorescent QPCR assay that can monitor gene copy number variation in cancer genomes (13, 14). Fluorescent QPCR has the following advantages over Southern blotting, FISH, and LOH studies: (*a*) both gene amplifications and deletions can be detected by fluorescent QPCR; (*b*) only a small amount of DNA is needed for this assay, so the small amounts of DNA that can be extracted from sources like serum and urine sediment are suitable for this assay; (*c*) only DNA (and not intact cells) is required for analysis, permitting analysis of acellular fluids, such as serum, that can still contain DNA from other sources; (*d*) no polymorphic markers are required for the assay, so all markers are informative in all individuals; and (*e*) pairing of an oncogene marker that is often amplified (*e.g.*, *erbB*-2) and a tumor suppressor marker that is often deleted (*e.g.*, *p16* or *p53*) maximizes the ability of the QPCR technique to detect quantitative alterations seen in cancer but not in normal cells. Here, we show that the sensitive and facile TaqMan PCR technique can detect *erbB*-2 amplifications in both tumors and sera of individuals with adenocarcinoma of the esophagus. Although such quantitative alterations occur only in a plurality of tumors, the study indicates a proof of principle that this technique is a valuable addition to the way cancer is monitored.

MATERIALS AND METHODS

Principle of TaqMan Assay. The TaqMan assay has been described previously (15–17). Briefly, the assay is based on the principle that successful PCR yields a fluorescent signal due to Taq-mediated exonuclease digestion of a fluorescence-labeled oligonucleotide homologous to a sequence between the two primers. The extent of digestion, which depends directly on the amount of PCR that occurs, can be quantified directly and accurately by measuring the increment in fluorescence that results from decreased energy transfer. This sensitive measurement allows detection in the exponential phase of the PCR, which is required for determination of initial genomic sequence copy number.

Mechanically, this was accomplished by performing the assay on an aliquot of the esophageal tumor or serum DNA. The

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[4] The abbreviations used are: FISH, fluorescent *in situ* hybridization; LOH, loss of heterozygosity; QPCR, quantitative PCR; nt, nucleotide(s).

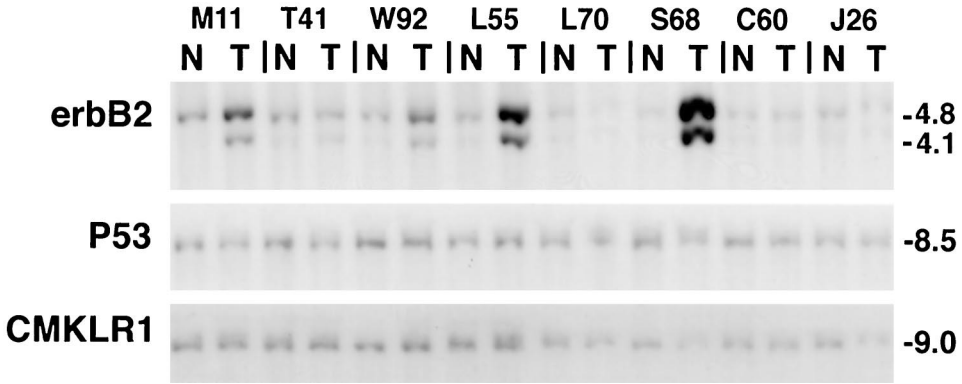

Fig. 1 Southern blot determination of *erbB*-2 and *p53* in esophageal cancer. Ten μg of DNA were obtained from esophageal tumor or normal control tissue (normal esophagus or gastric tissue). The resultant DNA was digested with *Eco*RI and separated on 0.9% agarose gels. The blots were probed, stripped, and rehybridized with probes for *erbB*-2, *p53*, and the control gene, *CMKLR1*.

relative gene copy number of two sequences was derived from the ratio of TaqMan PCR for each of these sequences. The oligonucleotides and primer for a given sequence were added to the reaction mixture containing DNA from the sample of interest in the presence of uracil DNA glycosylase (18) and AmpliTaq Gold (Perkin-Elmer Corp., Norwalk, CT), per the manufacturer's suggestion. Any carryover DNA was eliminated by uracil DNA glycosylase in a 2-min treatment at 50°C. Following a subsequent 10-min incubation at 95°C to denature the input DNA, the DNA was PCR-amplified using AmpliTaq Gold in a two-step amplification (15 s at 95°C to denature the DNA and 1 min at 60°C to permit annealing and elongation). To assay the initial concentration of the reactants (the gene copy number), we measured the number of cycles at which the reaction crosses a threshold value. This number, C_T, varied directly with the initial gene copy number. To measure the relative genomic copy numbers of two sequences in a given DNA sample, we divided the C_T derived using the first sequence with the given DNA sample by the C_T value using the second sequence. Derivation of this fraction was independent of DNA sample concentration, eliminating the requirement to measure DNA concentration accurately. As a result, an abnormal value of ≥ 2 could result from amplification of the first sequence and/or deletion of the second sequence. The contribution of each probe to the value could also be determined by evaluating additional probes, if desired, but this was not required for the TaqMan PCR assay.

DNA Preparation. DNA from normal leukocytes was purchased from Boehringer Mannheim. DNA from normal gastric tissue, normal esophagus, Barrett's mucosa, and esophageal adenocarcinoma (either Barrett's adenocarcinoma or adenocarcinoma arising from the cardia) were prepared as described (8). The normal and tumor tissues were removed from patients undergoing esophagectomy and designated by histological criteria as tumorous tissue, Barrett's metaplasia, normal-appearing gastric mucosa, and normal-appearing esophageal mucosa. Preparation of serum DNA was accomplished by a kit from Biotronics (Lowell, MA). This DNA was collected from patient samples obtained at the time of surgery for esophageal cancer, Barrett's mucosa with high-grade dysplasia, achalasia, or stricture.

PCR Primers for Measurement of Genomic Sequence Copy Number. The sequences of primers for *erbB*-2, *p53*, and *p16* were: (*a*) *erbB-2*: TaqMan probe, 6-FAM-5′-AGAGGGCCCTCTGCCTGCTGC-3′-TAMRA; erb2U18 (nt 252), 5′-AGGGAGTGGCAGAGACAC-3′; and erb2L17 (nt 528), 5′-GGCTGAAGGCAGGAGGA-3′; (*b*) *p53*: TaqMan probe, 6-FAM-5′-AGCAGCTCCTACACCGGCGGC-3′-TAMRA; p53U19 (nt 11,985), 5′-CCTGGTCCTCTGACTGCTC-3′; and p53L19 (nt 12,227), 5′-GTAGCTGCCCTGGTAGGTT-3′; and (*c*) *p16*: TaqMan probe, 6-FAM-5′-CTGCTGCTGCTCCACGGCGC-3′-TAMRA; p16U20 (nt 15), 5′-GGAAGCAAATGTAGGGGTAA-3′; and p16L17 (nt 267), 5′-CCAGCGTGTCCAGGAAG-3′.

Quantitative Southern Blotting. Quantitative Southern blot analysis of the *erbB*-2 and *p53* genes was performed as described previously (8). Southern blots were stripped after hybridization to erbB-2 or p53 and rehybridized with the single copy control gene, *CMKLR1* (19). The blots were quantified by scanning laser densitometry using an Image Quant Personal Densitometer (Molecular Dynamics, Sunnyvale, CA). The ratio of *erbB*-2/CMKLR1 or p53/CMKLR1 was used to quantitate *erbB*-2 or *p53* in normal, Barrett's metaplasia, and esophageal cancer samples.

RESULTS

Evaluation of TaqMan QPCR Systems for *erbB*-2, *p53*, and *p16* Markers. The resolution, sensitivity and reproducibility of TaqMan PCR was determined by performing TaqMan PCR on 2-fold dilution series of duplicated normal human DNA in the range from 20 ng to 24 pg. Three primer sets derived from the *erbB*-2, *p53*, and *p16* genes were tested in this analysis. The result confirmed that the TaqMan PCR values were halved as expected for each 2-fold dilution of the DNA. This was true down to 24 pg for the erbB-2 and p16 probes but only down to 1 ng for the p53 probe. This documents that the assay is sensitive enough to detect 2-fold concentration differences of DNA. The values duplicated for replicate samples performed for a given probe each time we ran the assay. With a maximum variation of 50% between duplicate points, the TaqMan PCR values yielded satisfactory results for each of the three probes with normal human DNA at every dilution.

We also checked the accuracy of TaqMan PCR by comparing TaqMan PCR results with previous Southern blotting. Using Southern blotting, we quantitated copy number of the oncogene, *erbB*-2, and the tumor suppressor gene, *p53*, in "nor-

Table 1 Copy number ratios in 39 normal samples and 39 tumor samples

Tissue source (no. of cases)	Taq Man PCR (*erbB*-2/*p53*)	Southern (*erbB*-2)
Barrett's metaplasia (2)		
1	0.8	
2	1.3	
Mean ± SD	1.1 ± 0.4	
Range	0.8–1.3	
Gastric, normal (9)		
1	0.9	
2	1.0	
3	1.0	
4	1.1	
5	1.1	
6	1.1	
7	1.1	
8	1.1	
9	1.2	
Mean ± SD	1.1 ± 0.1	
Range	0.9–1.2	
Esophagus, normal (28)		
1	0.7	
2	0.8	
3	0.8	
4	0.8	
5	0.8	
6	0.8	
7	0.8	
8	0.9	
9	0.9	
10	0.9	
11	0.9	
12	0.9	
13	0.9	
14	1.0	
15	1.0	
16	1.0	
17	1.0	
18	1.0	
19	1.1	
20	1.2	
21	1.3	
22	1.3	
23	1.4	
24	1.5	
25	1.7	
26	1.8	
27	1.8	
28	2.0	
Mean ± SD	1.1 ± 0.4	
Range	0.7–2.0	
Tumors (39)		
1	0.8	1
2	0.9	1
3	0.9	1
4	1.0	1
5	1.0	1
6	1.0	1
7	1.0	1
8	1.1	1
9	1.1	1
10	1.1	1
11	1.1	1
12	1.2	1
13	1.2	1
14	1.3	1
15	1.3	1
Tumor		
16	1.4	1
17	1.5	1
18	1.6	1
19	1.6	1
20	1.6	1
21	1.7	1
22	1.7	1
23	1.8	1
24	1.8	1
25	2.9 ± 0.3[a]	1.8
26	3.0 ± 0.4[a]	2.6
27	3.7 ± 0.2[a]	1
28	4.2 ± 0.2[a]	2.5
29	4.5 ± 0.6[a]	1
30	6.7 ± 0.2[a]	1
31	7.0 ± 2.7[a]	1
32	13.4 ± 1.8[a]	5.6
33	13.6 ± 1.6[a]	5.6
34	16.8	18.4
35	17.1	7.7
36	26.2	5.8
37	51.5	15.6
38	94.6 ± 9.2[b]	50.6
39	106.7 ± 17.6[b]	20

[a] Mean ± SD, two assays.
[b] Mean ± SD, four assays with dilution series.

mal" and "esophageal tumor" tissues (Fig. 1). The amount of *erbB*-2 or *p53* on the blots was normalized against the single copy gene, *CMKLR1*. Using TaqMan PCR, we then quantitated the ratio of the copy numbers of *erbB*-2/*p53* in these 39 normal samples and 39 tumor samples (Table 1).

The *erbB*-2/*CMKLR1*, *p53*/*CMKLR1* (by Southern blotting), and *erbB*-2/*p53* (by TaqMan PCR) ratios were ~1 (between 0.6 and 1.9) for controls. However, in tumors, amplifications of the oncogene *erbB-2* copy number relative to the tumor suppressor *p53* copy number were observed by both Southern blotting and TaqMan PCR (Table 1). Fifteen of the 39 tumors were abnormal, with a TaqMan PCR value of *erbB*-2/*p53* ≥2 (Table 1). For the lowest nine values among the TaqMan PCR values of ≥2 in Table 1, we repeated the TaqMan PCR analysis and confirmed that an amplification of *erbB*-2/*p53* of ≥2 was observed again. The means and SDs for these analyses are given (Table 1). In 11 of these 15 cases, the Southern blots also showed a copy number amplification of *erbB*-2. In four of the cases with smaller amplifications upon repeated TaqMan PCR, the Southern blots with *erbB*-2 did not detect an anomaly, consistent with the known difficulty using a single Southern blot to detect smaller quantitative alterations (10). The duplication of TaqMan PCR for these four samples with low degrees of *erbB*-2 amplifications verifies the increased sensitivity of TaqMan PCR to detect lower level amplifications. The ability of the TaqMan PCR technique to assay smaller amplifications that are ≥2-fold variations from the norm is substantiated by our extensive control data on both normal DNA and on dilutions of tumor DNA (Table 1; see above). These data

Table 2 Derivation by TaqMan PCR *erbB*-2/*p16* from serum and from tumors in individuals with esophageal cancer and abnormal serum values

Sample no.	Diagnosis[a]	No. of cases	TaqMan PCR	
			Serum	Tumor
Nonmalignant esophageal lesion		5	0.8–1.5 (1.1)[b]	
77	Achalasia, megaesophagus		0.8	
52	Stricture		1.0	
41	Staple exclusion		1.1	
58	Hiatal hernia		1.3	
73	Epiphrenic diverticulum		1.5	
Barrett's metaplasia of esophagus		5	0.8 –1.7 (1.2)[b]	
45	Barrett's, severe esophagitis		0.8	
64	Barrett's		0.9	
50	Barrett's with dysplasia		1.3	
67	Barrett's with high-grade dysplasia		1.6	
74	Barrett's with high-grade dysplasia		1.7	
Malignancy of esophagus (normal serum), pretreated with anticancer therapy		11	0.9–1.7[c]	0.4–1.8
55	III/adeno/cardia		0.9	0.4
53	III/squamous cell/lower		1.0	
70	III/adeno/cardia		1.1	0.6
66	IIB/adeno/middle		1.1	1.4
71	IIA/adeno/lower		1.1	1.4
44	IIA/adeno/lower		1.3	1.8
48	I/adeno/lower		1.3	0.9
54	I/adeno/lower		1.4	1.5
59	III/adeno/cardia		1.4	1.1
78	IIB/adeno/lower		1.7	
79	III/adeno/lower		1.7	0.4
Malignancy of esophagus (normal serum); not pretreated		14	0.7–1.6[c]	0.4–34.3[c]
80	IIB/adenol/cardia		0.7	34.3
47	IIA/adeno/cardia		0.9	1.8
46	I/adeno/lower		1.0	
69	I/adeno/cardia		1.0	1.0
42	III/adeno/lower		1.1	0.7
49	IIA/squamous cel/lower		1.1	1.4
56	IVA/adeno/cardia		1.1	
61	IIA/adeno/cardia		1.2	1.0
63	I/adeno/lower		1.2	
51	III/adeno/lower		1.4	1.6
65	IIB/adeno/cardia		1.4	0.5
43	IVA/adeno/cardia		1.5	0.8
75	III/adeno/lower		1.6	0.4
68	I/adeno/lower		1.6	
Malignancy of esophagus (abnormal serum), not pretreated		5	2.0–3.8[c]	2.5–8.9[c]
57	III/squamous cell/middle		2.0	2.5
60	I/adeno/cardia		2.2	7.9
72	III/adeno/lower		2.5	8.9
62	IIA/adeno/lower		3.7	3.1
76	IVA/adeno/lower		3.8	NA[d]

[a] Slashes represent stage/type/anatomic location. adeno, adenocarcinoma.
[b] Values represent ranges (means).
[c] Not including the tumor with a TaqMan PCR value of 34.3.
[d] NA, not available.

document the ability of the TaqMan PCR assay to distinguish 2-fold differences.

Detection of Abnormality in the Serum DNA from Esophageal Adenocarcinoma Patients. Given the small amount of DNA required for TaqMan PCR, we applied this technique to the analysis of DNA in serum. There was significantly less DNA in serum than available from tumor samples, averaging ~100 copies per reaction. At these low concentrations of DNA, our *erbB*-2 probe was appropriately sensitive for TaqMan PCR, but the *p53* probe was not. The probe for p16, however, was sufficiently sensitive to work at these low DNA concentrations. As noted above, this probe satisfied all of the requirements met by the *erbB*-2 probe, showing a good 2-fold discrimination between DNA samples at low concentrations, down to 24 pg.

Therefore, we used the probe pairing of *erbB*-2 and *p16* to screen 39 sera blindly (Table 2; these sera were from different patients than the tumor DNAs that were analyzed in Table 1 for

erbB-2/*p53*). We also did the corresponding tumor DNA with *erbB*-2 and *p16* when the tumor was available. In five cases, the esophageal lesion was nonmalignant, and in another five cases, the sera was from patients with premalignant Barrett's metaplasia or Barrett's metaplasia with dysplastic changes. In each of these 10 cases, the serum TaqMan PCR was normal (<2). We detected amplifications ≥ 2 of the *erbB*-2/p16 ratio in five serum samples from 19 subjects with untreated esophageal tumors. Presumably, all five abnormal serum samples corresponded to an untreated tumor with *erbB*-2 amplification (in four, the tumor showed an amplification of *erbB*-2; and in one, the tumor was unavailable but presumably had such an amplification). There were two tumors with normal serum *erbB*-2/*p16* ratios that showed a deletion equivalent to one copy of *erbB*-2, and one tumor that showed a large amplification of *erbB*-2. In neither case did we see a corresponding quantitative anomaly of serum *erbB*-2/*p16*. In addition, we did not observe anomalies of the serum in the remaining 11 samples from subjects who had been treated by radiotherapy and/or chemotherapy prior to obtaining the sample (two of whom showed a deletion of *erbB*-2/*p16* in the tumor).

DISCUSSION

Accuracy of the TaqMan Methodology. Although competitive PCR technologies have been advanced to perform quantitation (20, 21), they are difficult to perform with great facility and accuracy. In contrast, the facile TaqMan PCR technology (15–17) affords a real-time assay. Our extensive control data permit the demarcation of abnormal TaqMan PCR values to be ≥ 2. When the TaqMan PCR technique was applied to normal leukocyte DNA, the variation we observed was routinely $<50\%$ between replicate samples. In no case did we observe a TaqMan PCR value for this normal DNA of <0.7 or >1.3 times the expected value. Furthermore, only 5 of 39 esophageal samples that were classified as normal by histological criteria had TaqMan PCR values between 1.4 and 1.9 (*i.e.*, showed a deviation of $>50\%$ from a normal value of 1; the single normal sample with a value of 2.0 came from an esophagus in which the tumor had a very high value of 16.8). Indeed, we saw a lower range of high TaqMan PCR values with known normal standard DNA than DNA from these normal esophageal samples taken from subjects with known esophageal cancer. These high TaqMan PCR values could reflect contamination of normal cells with malignant cells, small increases of gene copy number in premalignant cells. or unsuspected difficulties arising during DNA purification.

In all 11 cases in which Southern blotting detected an amplification of *erbB*-2, the TaqMan PCR value was abnormally elevated. However, the amplifications seen on the Southern blot for *erbB*-2 were frequently less than the changes seen in TaqMan PCR (Table 1). Given the reproducibility of TaqMan PCR, the most likely explanation for this discrepancy is that Southern blotting is less sensitive and underestimates the true value of *erbB*-2 amplification. In particular, there were four cases in which the TaqMan PCR values were abnormally elevated but the Southern blots did not show quantitative changes. The effect of p53 deletion could not fully explain the difference in these four cases. This apparent insensitivity of the Southern blotting method reflects the difficulty of this method to detect small variations without multiple repetitions (10). A similar determination that TaqMan PCR represents a suitable methodology to ascertain amplifications comes from two recent analyses of amplification events in breast cancer (22, 23). In summary, compared with Southern blotting, TaqMan PCR has the dual advantages of increased sensitivity and the ability to use both deletions and amplifications because our usage depends on the ratio between a tumor suppressor gene and an oncogene (Table 1).

Analysis of Serum for Monitoring the Patient with Cancer. A major contribution of this analysis is the potential to detect amplifications in serum that result from cancer. Stroun *et al*. (24) showed that there was a small amount of free DNA in serum due to death of normal nucleated blood cells and/or vessel wall endothelial cells. The lysed genome of tumor cells that outstrip their blood supply and/or represent the products of metastatic cells are added to this serum DNA content from normal cells. On the basis of this, a variety of tumors can yield LOH or novel restriction enzymes digestion sites detectable in serum DNA (1–5). The finding that tumor DNA was sometimes present in the serum led us to detect serum DNA quantitative alterations by TaqMan PCR. The sensitivity of TaqMan PCR coupled with the lack of requirement for intact cells (as in FISH) could make this a worthwhile approach to monitoring the serum for both detection and following therapy of cancer.

Analysis of serum with the probes *erbB*-2 and *p16* is detailed in Table 2. For this task, our p53 probe was not sufficiently sensitive, so we had to use our p16 probe instead to monitor the small amount of DNA in serum along with the *erbB*-2 probe. All 10 sera from subjects who did not have esophageal cancer had normal serum QPCR values of <2, as expected. All 11 sera from patients who had preoperative chemotherapy and/or radiotherapy, in whom tumor size would be expected to be minimal, did not show abnormal serum values. Five sera of 19 from individuals with esophageal tumors that were not pretreated, including 5 of 6 sera with corresponding elevated tumor values of the *erbB*-2/*p16* ratio, were abnormally elevated; *i.e.*, as they showed TaqMan PCR values of ≥ 2. Thus, five of six untreated tumors could be detected by serum analysis, reflecting bulky tumors outstripping their blood supply and/or hematogenous dissemination (1–5). Presumably, the one tumor with an elevated *erbB*-2/*p16* ratio that did not show an elevated serum *erbB*-2/*p16* ratio had not seeded the serum. Our results indicated that TaqMan PCR could be used to monitor cancer therapy by serum analyses. Although this series is too small to draw definite conclusions, it is of interest to note that the serum was positive with tumors of different stages. Although the ultimate clinical significance will require larger studies, this suggests the feasibility of monitoring serum by the facile QPCR technology.

The inability to detect a loss of erbB-2/p16 and the finding of cases in which the serum QPCR values are less than the tumor QPCR values both indicate that normal cellular DNA in the serum (24) can dilute esophageal tumor DNA in the serum. In this light, screening for QPCR anomalies with greatest quantitative differences will maximize the sensitivity of this test. The failure to find LOH anomalies in serum of colon cancer subjects

(5) underscores that the phenomenon of serum abnormalities in cancers can be tumor specific.

In the tumor, normal DNA is contributed by normal cells in the biopsy specimen; in the serum, normal DNA is contributed by lysis of normal blood and endothelial cells. This explains why there is discordance between tumor and serum QPCR values even when hematogenous seeding by tumor cells has occurred. Thus, although this study underscores the potential usefulness of using the simple, rapid, and inexpensive technique of TaqMan PCR to monitor serum, questions regarding the sensitivity of the technique in this and other malignancies remain to be addressed. Although this represents only a small series of relevant patients who were not pretreated and who had significant amplification of *erbB*-2, it indicates the potential usefulness of monitoring QPCR in serum of patients with amplifications of QPCR in the tumor.

REFERENCES

1. Nawroz, H., Koch, W., Anker, P., Stroun, M., and Sidransky, D. Microsatellite alterations in serum DNA of head and neck cancer patients. Nat. Med., *2:* 1035–1037, 1996.

2. Chen, X. Q., Stroun, M., Magnenat, J., Nicod, L. P., Kurt, A., Lyautey, J., Lederrey, C., and Anker, P. Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nat. Med., *2:* 1033–1035, 1996.

3. Goessl, C., Heicappell, R., Munker, R., Anker, P., Stroun, M., Krause, H., Muller, M., and Miller, K. Microsatellite analysis of plasma DNA from patients with clear cell renal carcinoma. Cancer Res., *58:* 4728–4732, 1998.

4. Sanchez-Cespedes, M., Monzo, M., Rosell, R., Pifarre, A., Calvo, R., Lopez-Cabrerizo, M. P., and Astudillo, J. Detection of chromosome 3p alterations in serum DNA of non-small-cell lung cancer patients. Ann. Oncol., *9:* 113–116, 1998.

5. Hibi, K., Robinson, C. R., Booker, S., Wu, L., Hamilton, S. R., Sidransky, D., and Jen, J. Molecular detection of genetic alterations in the serum of colorectal cancer patients. Cancer Res., *58:* 1405–1407, 1998.

6. Reid, B. J. *In:* Y. Yamada, D. H. Alpers, C. Owyang, D. W. Powel, and F. E. Silverstein (eds.), Textbook of Gastroenterology, pp. 1159–1178. Philadelphia: J. B. Lippincott, 1991.

7. Blot, W. J., Devesa, S. S., Kneller, R. W., and Fraumeni, J. F. Rising incidence of adenocarcinoma of the esophagus and gastric cardia. J. Am. Med. Assoc., *265:* 1287–1289, 1991.

8. Al-Kasspooles, M., Moore, J. H., Orringer, M. B., and Beer, D. G. Amplification and overexpression of the EGFR and *erbB*2 genes in human esophageal adenocarcinomas. Int. J. Cancer, *54:* 213–219, 1993.

9. Southern, E. M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol., *98:* 503–517, 1975.

10. Korenberg, J. R. Chen, X. N., Schipper, R., Sun, Z., Gonsky, R., Gerwehr, S., Carpenter, N., Daumer, C., Dignan, P., Disteche, C., Graham, J. M., Hugdins, L., McGillivray, B., Miyazaki, K., Ogasawara, N., Park, J. P., Pagon, R., Pueschel, S., Sack, G., Say, B. Schuffenhauer, S., Soukup, S., and Yamanaka, T. Down syndrome phenotypes: the consequences of chromosomal imbalance. Proc. Natl. Acad. Sci. USA, *91:* 4997–5001, 1994.

11. van Ommen, G. J., Breuning, M. H., and Raap, A. K. FISH in genome research and molecular diagnostics. Curr. Opin. Genet. Dev., *5:* 304–308, 1995.

12. Lasko, D., Cavenee, W., and Nordenskjold, M. Loss of constitutional heterozygosity in human cancer. Annu. Rev. Genet., *25:* 281–314, 1991.

13. Chiang, P-W., Song, W-J., Wu, K-Y., Korenberg, J. R., Fogel, E. J., Van Keuren, M. L., Lashkari, D., and Kurnit, D. M. Use of a fluorescent PCR reaction to detect genomic sequence copy number and transcriptional abundance. Genome Res., *6:* 1013–1026, 1996.

14. Cairns, J. P., Chiang, P-W., Ramamoorthy, S., Kurnit, D. M., and Sidransky, D. A comparison between microsatellite and quantitative-PCR analyses to detect frequent p16 copy number changes in primary bladder tumors. Clin. Cancer Res., *4:* 441–444, 1998.

15. Higuchi, R., Dollinger, G., Walsh, P. S., and Griffith, P. R. Simultaneous amplification and detection of specific DNA sequences. Biotechnology, *10:* 413–417, 1992.

16. Livak, K. J., Flood, S. J. A., Marmaro, J., Giusti, W., and Deetz, K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl., *4:* 357–362, 1995.

17. Heid, C. A., Stevens, J., Livak, K. J., and Williams, P. M. Real time quantitative PCR. Genome Res., *6:* 986–994, 1996.

18. Rashtchian, A., Buchman, G. W., Schuster, D. M., and Berninger, M. S. Uracil DNA glycosylase-mediated cloning of polymerase chain reaction-amplified DNA. Application to genomic and cDNA cloning. Anal. Biochem., *206:* 91–97, 1992.

19. Gantz, I., Konda, Y., Yang, Y. K., Miller, D. E., Dierick, H. A., and Yamada, T. Molecular cloning of a novel receptor (CMKLR1) with homology to the chemotactic factor receptors. Cytogenet. Cell Genet., *74:* 286–290, 1996.

20. Lubin, M. B., Elashoff, J. D., Wang, S. J., Rotter, J. I., and Toyoda, H. Precise gene dosage determination by polymerase chain reaction: theory, methodology, and statistical approach. Mol. Cell. Probes, *5:* 307–317, 1991.

21. Celi, F. S., Cohen, M. M., Antonarakis, S. E., Wertheimer, E., Roth, J., and Shuldiner, A. R. Determination of gene dosage by a quantitative adaptation of the polymerase chain reaction (gd-PCR): rapid detection of deletions and duplications of gene sequences. Genomics, *21:* 304–310, 1994.

22. Gelmini, S., Orlando, C., Sestini, R., Vona, G., Pinzani, P., Ruocco, L., and Pazzagli, M. Quantitative polymerase chain reaction-based homogeneous assay with fluorogenic probes to measure c-*erbB*-2 oncogene amplification. Clin. Chem., *43:* 752–758, 1997.

23. Bieche, I., Olivi, M., Champeme, M. H., Vidaud, D., Lidereau, R., and Vidaud, M. Novel approach to quantitative polymerase chain reaction using real-time detection: application to the detection of gene amplification in breast cancer. Int. J. Cancer, *78:* 661–666, 1998.

24. Stroun, M., Anker, P., Lyautey, J., Lederrey, C., and Maurice, P. A. Isolation and characterization of DNA from the plasma of cancer patients. Eur. J. Cancer Clin. Oncol., *23:* 707–712, 1987.

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