Appendix S1

Cell culture. All cell lines (HCC38, HCC70, SKBR3, HCT116) were originally purchased from the American Type Culture Collection and maintained in RPMI-1640 cell culture medium. All cell culture media were supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin and cells were incubated at 37°C with 5% CO_2.

Sensitivity of the mutation-specific ddPCR assay. To confirm the sensitivity to the presence of specific mutations (*PIK3CA* H1047R, *TP53* R175H and R248Q), we performed preliminary experiments using genomic DNA extracted from cell lines (Fig. S4). Mutant and wild type cell lines in each assay are shown in Table SIII. The mutant fragment was mixed with wild-type DNA in ratios of 1:1 (50%), 1:9 (10%), 1:19 (5%), 1:99 (1%), 1:199 (0.5%), 1:999 (0.1%), 1:9999 (0.01%). In any of the assays, it was confirmed that mutation could be detected even with a small amount of 0.01% of the mutant type gene as the wild type. Percentage mutant (% mutant) was calculated from generated Poisson concentrations as follows: % mutant = (FAM)/(FAM + HEX) x 100. FAM refers to 6-fluorescein amidite and HEX to hexachloro-fluorescein.

Figure S1. Representative images of pAKT and pS6RP staining. Representative microscopy images of tumor cells (original magnification, x100) showing (A) strong staining of pAKT, (B) moderate staining of pAKT, (C) weak staining of pAKT, (D) no staining of pAKT, (E) strong staining of pS6RP, (F) moderate staining of pS6RP, (G) weak staining of pS6RP and (H) no staining of pS6RP.

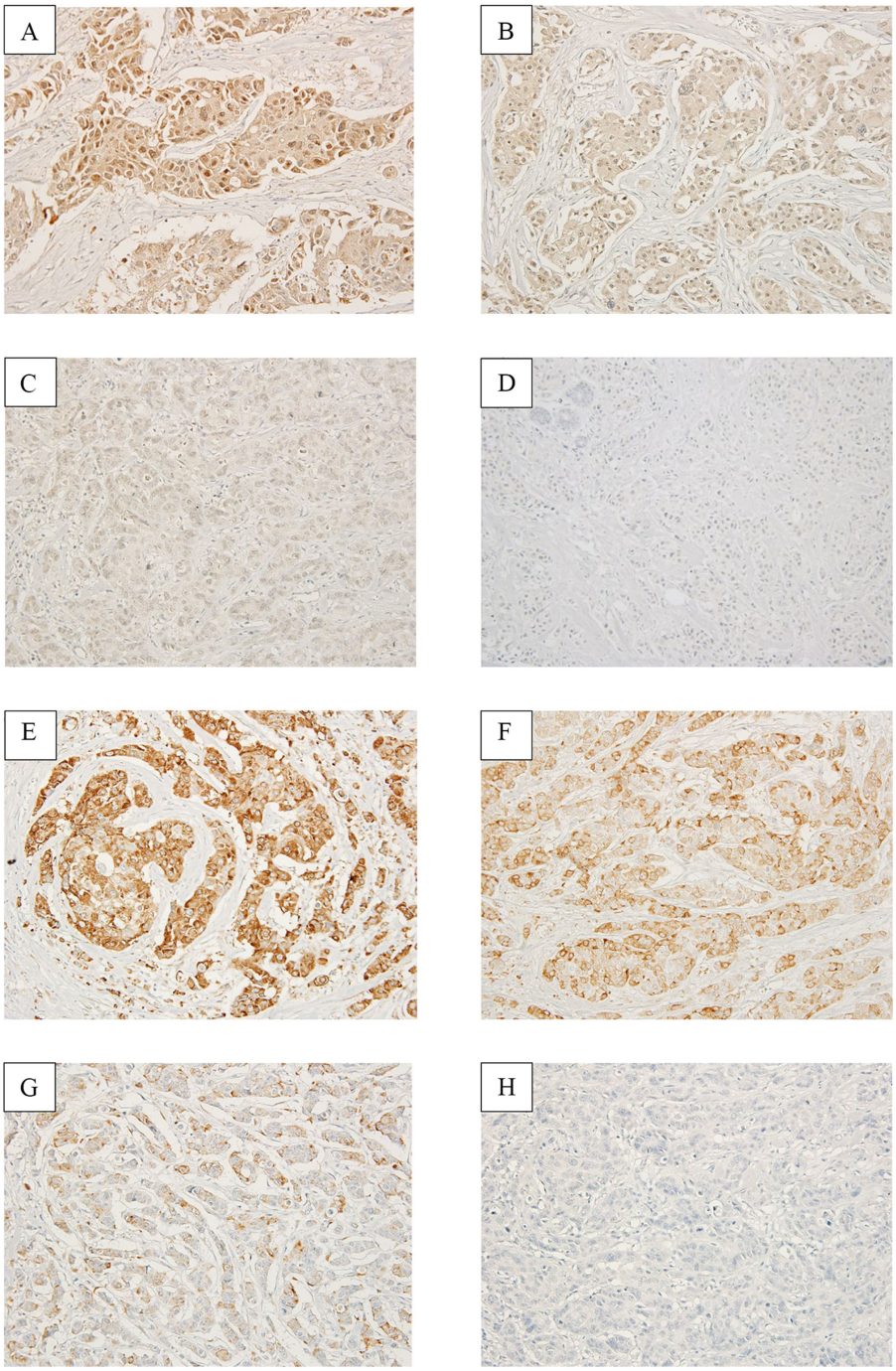

Figure S2. Summary of variant call processing.

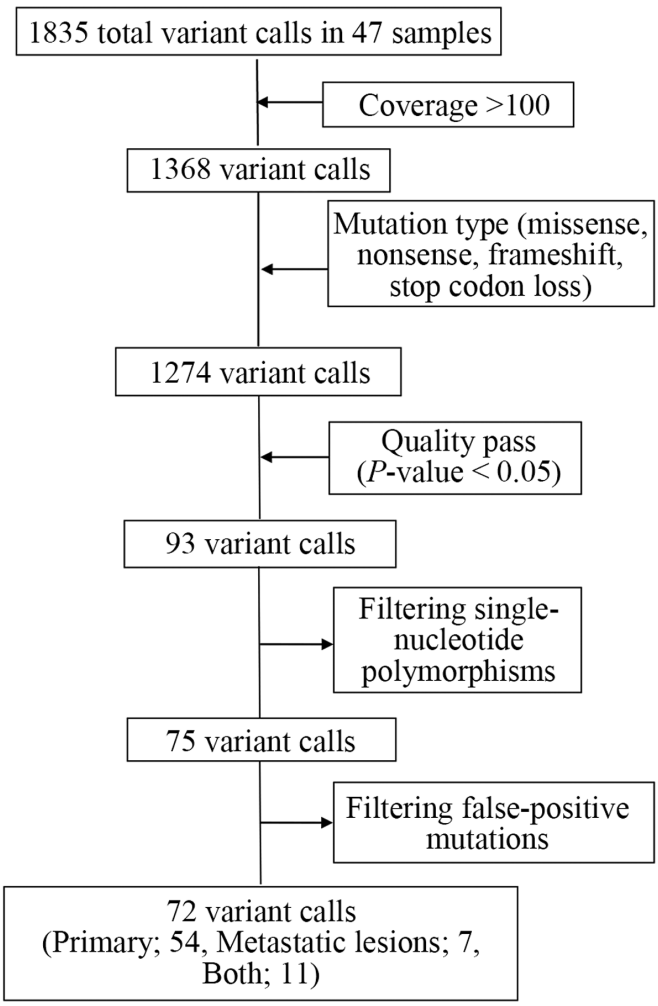

Figure S3. Comparison of mutation frequencies in 11 primary and 13 metastatic tumors in each of the cases.

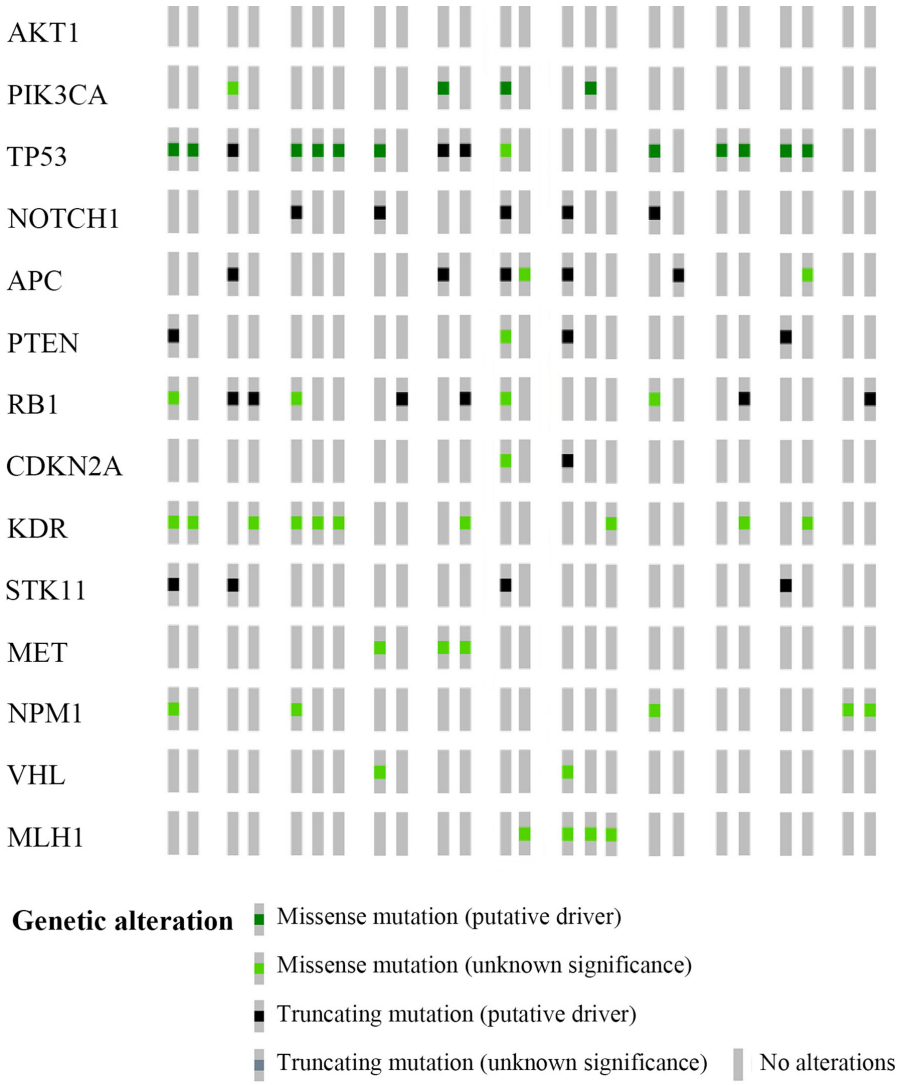

Figure S4. Sensitivity of the mutation-specific ddPCR assay. To confirm the sensitivity of the specific mutations (*PIK3CA* H1047R; *TP53* R175H and R248Q), we performed preliminary experiments using genomic DNA extracted from cell lines (Appendix S1). Allele frequencies of each mutant were confirmed by ddPCR with limiting dilutions from 50% to 0.01%. (A) Allele frequency (fractional abundance) of PIK3CA. (B) Allele frequency of TP53 R175H. (C) Allele frequency of TP53 R248Q. In our experimental system, all mutations were detectable with a sensitivity of 0.01%.

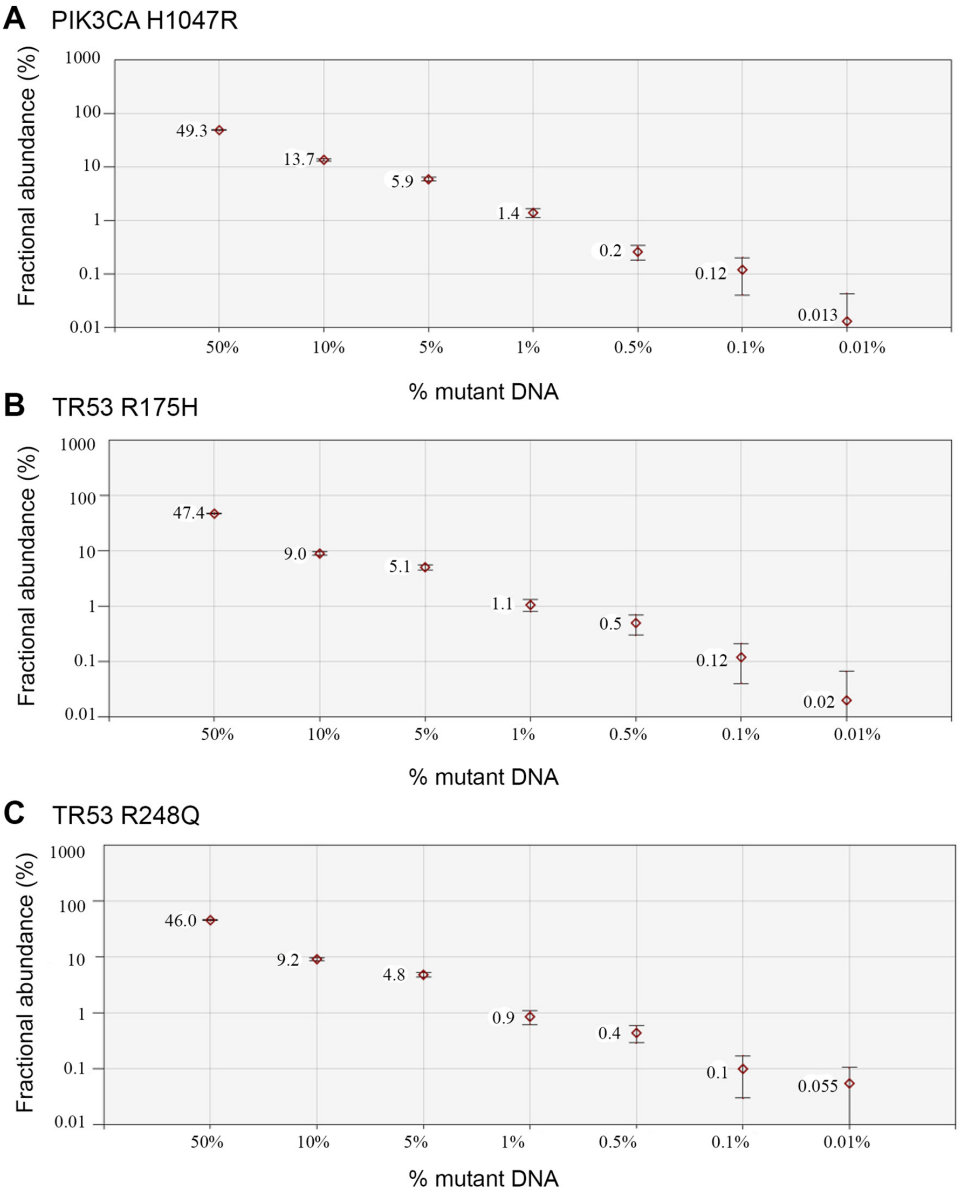

Figure S5. Clinical course of case reports. (A) A 78-year-old woman with a diagnosis of TNBC. The graph shows the change in the percentage of *PIK3CA* H1047R mutation detected in cfDNA using ddPCR and the CEA levels during the clinical course after surgery. Before radiographical recurrence detection, the *PIK3CA* H1047R in cfDNA was positive, while the tumor marker was marginal. (B) A 62-year-old woman diagnosed with TNBC. The graph shows the change in the percentage of *AKT1* E17K mutation detected in cfDNA using ddPCR and the CA15-3 levels during the clinical course after surgery. Both the AKT1 E17K in cfDNA and CA15-3 were positive before radiographical recurrence detection. (C) A 85-year-old woman with a diagnosis of TNBC. The graph shows the change in the percentage of *PIK3CA* H1047R mutation detected in cfDNA using ddPCR and the CEA levels during the clinical course after surgery. When the brain metastasis occurred, the PIK3CA H1047R in cfDNA was positive, while the tumor marker was negative. (D) A 47-year-old woman diagnosed with TNBC. The graph shows the change in the percentage of *PIK3CA* H1047R mutation detected in cfDNA using ddPCR and the CEA levels during the clinical course after surgery. The patient maintains no recurrence after resection of recurrent axillary lymph nodes, was positive for tumor marker but the *PIK3CA* H1047R in cfDNA was negative. CT or MRI imaging revealed no recurrence which consistent with cfDNA results. cfDNA, cell-free DNA; ddPCR, droplet digital polymerase chain reaction; CT, computed tomography; CA15-3, carbohydrate antigen 15-3; CEA, carcinoembryonic antigen

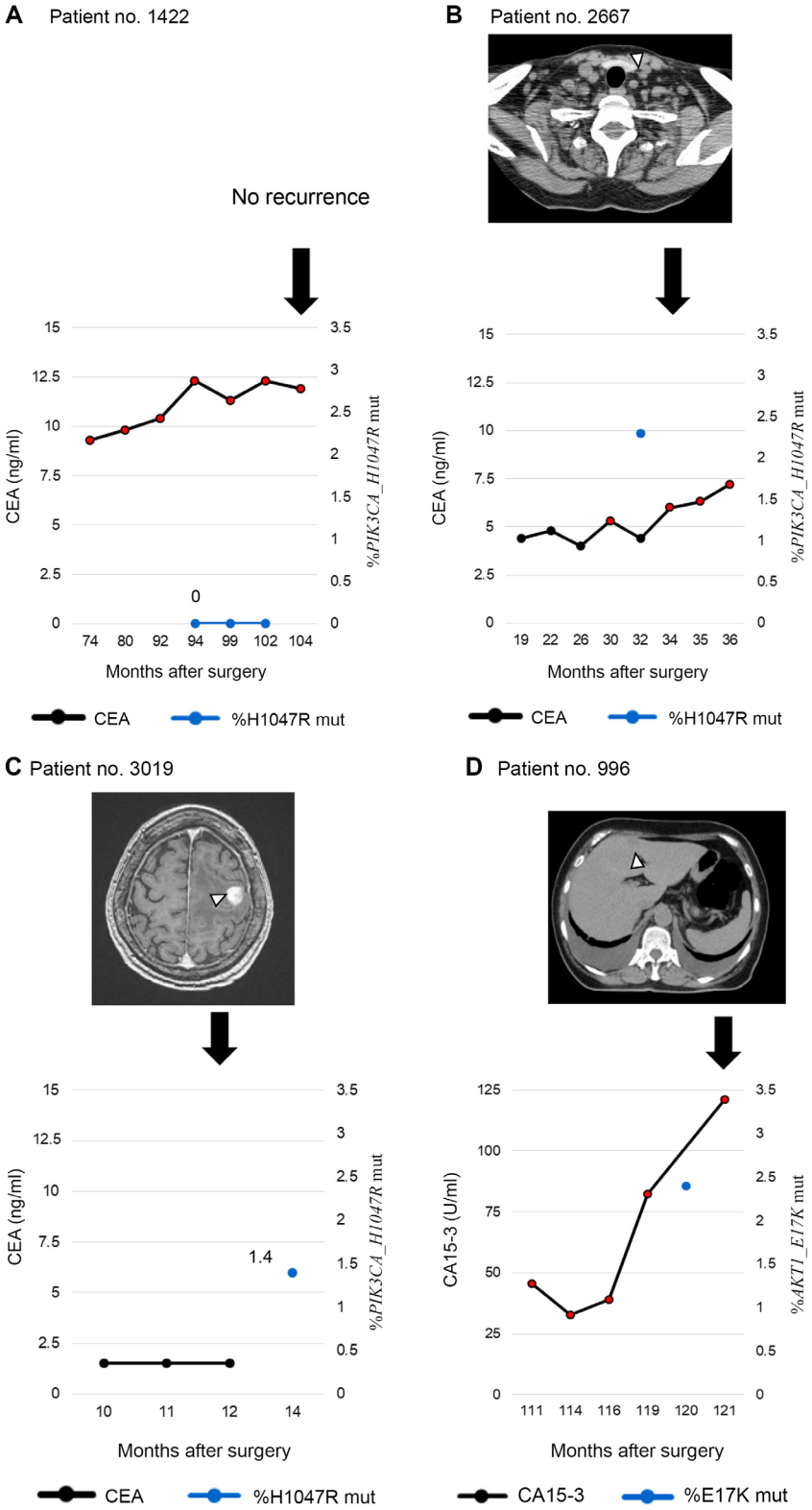

Table SI. List of primers and probes for specific ddPCR assays.

Primers and probes	Sequence	Product size (bp)
Primers		
Forward *PIK3CA*	5'-AGCAAGAGGCTTTGGAGTATTT-3'	102
Reverse *PIK3CA*	5'-CATGCTGTTTAATTGTGTGGAAGA-3'	
Forward *TP53* (for R175H)	5'-CAGCACATGACGGAGGTT-3'	100
Reverse *TP53* (for R175H)	5'-TTCCTTCCACTCGGATAAGATG-3'	
Forward TP53 (for R248Q)	5'-ACAACTACATGTGTAACAGTTCCT-3'	80
Reverse TP53 (for R248Q)	5'-GGAGTCTTCCAGTGTGATGATG-3'	
Probes		
Wild-type *PIK3CA* (HEX)	5'-HEX-ACC+ATG+A+T+GT+GC+AT-IowaBlack FQ-3'	
Mutant *PIK3CA* H1047R (FAM)	5'-6FAM-CCA+TG+A+C+GT+GCAT-IowaBlack FQ-3'	
Wild-type *TP53* (HEX)	5'-HEX-CA+G+C+GC+CTCA-IowaBlack FQ-3'	
Mutant *TP53* R175H (FAM)	5'-6FAM-CA+G+T+GC+CT+CA-IowaBlack FQ-3'	
Wild-type *TP53* (HEX)	5'-HEX-CCT+C+C+GG+T+TC-IowaBlack FQ-3'	
Mutant *TP53* R248Q (FAM)	5'-6FAM-CCT+C+T+GGT+T+CA-IowaBlack FQ-3'	

+N represents LNA base. ddPCR, droplet digital polymerase chain reaction; LNA, locked nucleic acid.

Table SII. Association between *AKT1* E17K/*PIK3CA* H1047R mutations (by NGS and ddPCR) and pAKT/pS6RP expression (using IHC).

Protein	IHC score	*PIK3CA* and *AKT1* mutant no. (%)	*PIK3CA* and *AKT1* wild no. (%)	P-value
pAKT	0	1 (10)	0	0.044[a]
	1	0	6 (16.2)	
	2	5 (50)	29 (78.4)	
	3	4 (40)	2 (5.4)	
pS6RP	0	0	4 (10.8)	0.105
	1	1 (10)	1 (2.7)	
	2	2 (20)	18 (48.6)	
	3	7 (70)	14 (37.8)	

NGS, next-generation sequencing; ddPCR, droplet digital polymerase chain reaction; IHC, immunohistochemistry; pAKT, phospho-AKT; pS6RP, phospho-S6RP. [a]P<0.05. To assess IHC score differences between *PIK3CA* and *AKT1* mutant samples and wild-type samples, the median scores for the mutant and wild type groups were determined and compared by Mann-Whitney U test.

Table SIII. Cell line and genetic mutations.

Target	*PIK3CA* H1047R	*TP53* R175H	*TP53* R248Q
Mutant cell line	HCT116	SKBR-3	HCC70
Wild type cell line	HCC38	HCC38	HCC38