

学位論文

Intertumor and intratumor heterogeneity of *PIK3CA* mutations in extramammary Paget's disease.

(乳房外パジェット病における PIK3CA 遺伝子変異の腫瘍間および腫瘍内不均一性)

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Original article

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Intertumor and intratumor heterogeneity of *PIK3CA* mutations in extramammary Paget's disease.

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Short running title: *PIK3CA* mutations in EMPD

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Abstract

Although the prognosis of patients with Extramammary Paget's disease (EMPD) treated with radical resection is good, the prognosis of EMPD with distant metastasis is very poor.

PIK3CA mutations predict a good response to *PIK3CA* inhibitors. The aim of this study was to investigate the occurrence rate of *PIK3CA* mutations (including multiple mutations [MMs]) related to the intertumor and intratumor heterogeneity in EMPD and to evaluate the correlation between these mutations and clinical parameters of EMPD. We performed droplet digital polymerase chain reaction to detect *PIK3CA* mutations (E542K, E545K, H1047R and MMs) in 68 patients with EMPD. In addition, we investigated the presence of *PIK3CA* mutations at multiple sites in 16 patients with *PIK3CA* mutations to assess the intratumor heterogeneity of *PIK3CA* mutations in EMPD. The frequency of one or more *PIK3CA* mutations in patients with EMPD was 30.8% (21/68). The frequency of E542K, E545K, and H1047R, and MMs were 10.2% (7/68), 13.2% (9/68), 11.7% (8/68), and 4.4% (3/68), respectively. No significant correlation was found between *PIK3CA* mutation patterns and clinical parameters. Of the 21 patients with *PIK3CA* mutations, 16 patients with tissue samples that could be analyzed at multiple sites were examined. The proportion of patients with the same *PIK3CA* mutations at all sites was 12.5% (2/16). The proportion of patients with the same *PIK3CA* mutations at least two or more sites, but not

at all sites, was 31.2% (5/16). The proportion of patients with no *PIK3CA* mutations at other sites was 37.5% (6/16). The proportion of patients with other *PIK3CA* mutations at other sites was 18.7% (3/16). There is intertumor and intratumor heterogeneity of *PIK3CA* mutations. *PIK3CA* mutations in EMPD may be progressor mutations in EMPD.

Key words.

PIK3CA, multiple mutations, intertumor heterogeneity, intratumor heterogeneity, droplet digital PCR

Introduction

Extramammary Paget's disease (EMPD) is a rare skin malignant skin adenocarcinoma that occurs mainly in pubic or axillary lesions. Although the prognosis of patients treated with radical resection is good, the prognosis of EMPD with distant metastasis is very poor. Recently, several therapeutic molecules have been identified. CDK4¹, EpCAM², CA125³ and JAK2⁴, for which inhibitors already exist, are overexpressed in EMPD, and the prognosis of patients with strong a staining intensity of CA125 and JAK2 tend to be poor^{3,4}. The analysis of exome and targeted sequencing revealed that several driver mutations, such as mutations in TP53 and ERBB2, are involved in the pathogenesis of EMPD⁵⁻⁹. Based on these findings, there is a need to investigate further therapeutic targets and their clinical significance in EMPD.

Oncogenic *PIK3CA* mutation activates the phosphoinositide 3-kinase (PI3K) enzyme, and PI3K-AKT signaling activation induces several growth-regulatory transcription factors¹⁰. Approximately 80% of human cancers with *PIK3CA* mutations are observed in 'hot spot' regions such as exon 9 (E542K and E545K) and exon 20 (H1047R)^{11,12}. *PIK3CA* mutations predict a good response to PI3K inhibitors¹³. In addition, multiple driver mutations (MMs) in individual genes have been observed in various types of cancer, and frequent genes with MMs are *PIK3CA* and *EGFR*¹⁴. In an in

vitro study, multiple *PIK3CA* mutations accelerated downstream PI3K-AKT signaling, and cancer cells with *PIK3CA* MMs were strongly inhibited by PI3K inhibitors¹⁴.

The occurrence rate of *PIK3CA* mutations in EMPD is approximately 5-35%^{5-9,15,16}. Although Kang Z et al. reported that *PIK3CA* mutations correlate with invasive EMPD¹⁵, Stasenko et al reported that all EMPD tissues with *PIK3CA* mutations are non-invasive⁷. Only two reports have shown that the frequency of MMs of *PIK3CA* in EMPD is 2-3%^{8,15}.

Intertumor heterogeneity is defined as genetic variation between individuals with the same tumor type, while intratumor heterogeneity is defined as subclonal diversity within a single tumor¹⁷. However, the clinical significance of genetic intertumor heterogeneity, including *PIK3CA* mutations, in a large case series of EMPD, remains unclear. Genetic intratumor heterogeneity in EMPD has not yet been reported, although intratumor heterogeneity has attracted attention as a source of diversity in genomic variation in several cancers, including melanoma^{18,19}.

Thus, the purpose of our study was to investigate the occurrence rate of *PIK3CA* mutations (including MMs) related to the intertumor and intratumor heterogeneity in EMPD and to evaluate the correlation between these mutations and clinical parameters of EMPD.

Materials and methods

Patients

Skin tissues were obtained from a total of 68 patients with EMPD treated between January 2006 and September 2019 at Kumamoto University Hospital. The institutional review board of Kumamoto University approved this study, and written informed consents was obtained from all patients in accordance with the principles of the Declaration of Helsinki.

Purification of genomic DNA and droplet digital polymerase chain reaction analysis

Formalin-fixed paraffin-embedded (FFPE) tissue sections were cut at a thickness of 8 μ m, and the area containing 80% of EMPD cells was excised with a scalpel and referred to as hematoxylin and eosin-stained specimens. Ten to twenty sections were used as samples for DNA extraction. DNA was isolated using a QIAmp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), and DNA quality was assessed using agarose gel electrophoresis and absorbance. The DNA fragment sizes ranged from 300 bp to 2000 bp (Supplementary Figure 1), and the A260 / A280 ratio of the samples was above 1.8. The amount of DNA for ddPCR was approximately 130ng in accordance with the manufacturer's protocol, and ddPCR was performed using a QX200 droplet digital PCR system (Bio-Rad, Berkeley, CA, USA) as previously reported²⁰. ddPCR probes for *PIK3CA* mutations (E542K/E545K/H1047R) were purchased from Bio-Rad. To determine the cut off value

of *PIK3CA* mutations, we assessed the false positive rate using *PIK3CA* Wild Type Reference Standard (Horizon Discovery, Cambridge, United Kingdom) as negative control. ddPCR showed that the false positive rate ranged from 0.04 to 0.09% of each *PIK3CA* mutations (Supplemental Figure 2). However, following the results of similar experiments in a previous study²¹, which showed that the false positive rate was 0.5%, we set the cutoff of variant allele frequency (VAF) at 1%.

Statistical analysis

Fisher exact test was used to discriminate clinical parameters (sex, degree of invasion, lymph node metastasis, distant organ metastasis) of the positive and negative groups of each *PIK3CA* mutation (E542K, E545K, H1047R and MMs) in EMPD patients. The relationship between age and the presence/absence of *PIK3CA* mutations was analyzed using the Mann–Whitney U test. Comparison of survival rates between the two groups was conducted using Kaplan-Meier survival analysis. Statistical analyses were performed using GraphPad Prism version 7 (GraphPad Software, La Jolla, CA, USA). Statistical significance was set at $p < 0.05$.

Results

*Intertumor heterogeneity of *PIK3CA* mutations in EMPD*

To evaluate the clinical significance of *PIK3CA* mutations in EMPD, we conducted

ddPCR using tumor tissues from 68 patients with EMPD (Figure 1, Supplemental Figure 3 and Table 1). The frequency of one or more *PIK3CA* mutations in EMPD was 30.8% (21/68). The frequencies of E542K, E545K and H1047R *PIK3CA* mutations were 10.2% (7/68), 13.2% (9/68), and 11.7% (8/68), respectively. The frequency of MMs of *PIK3CA* in EMPD was 4.4% (3/68, case 9 [E542K, E545K], 21 [E542K, E545K], and 45 [E545K, H1047R]). No significant correlation was found between *PIK3CA* mutations (total, E542K, E545K, H1047R, and MMs) and clinical parameters (age, sex, degree of invasion, lymph node metastasis, distant metastasis, and survival rate, Table 2, Supplementary Figure 6).

Intratumor heterogeneity of PIK3CA mutations in EMPD

Next, to assess the intratumor heterogeneity of *PIK3CA* mutations in EMPD, we investigated the presence of *PIK3CA* mutations at multiple sites. Of the 21 patients in whom one or more *PIK3CA* mutations was detected, 16 patients with tissue samples that could be analyzed at multiple sites were examined (Figure 2, Supplemental Figure 4). The proportion of patients with the same *PIK3CA* mutations in all sites was 12.5% (2/16; case 18 and 32). The proportion of patients with the same *PIK3CA* mutations at least two or more sites, but not all sites, was 31.2% (5/16; case 9, 37, 41, 49 and 53). The proportion of patients with no *PIK3CA* mutations at other sites was 37.5% (6/16; case 2, 8, 21, 50,

56, and 65). The proportion of patients with other *PIK3CA* mutations at other sites was 18.7% (3/16; case 3, 17, and 19). Besides, there were no differences in the cell morphology between mutation-positive and -negative areas in each patients (Figure 2). In case 2, tumor tissues with postoperative recurrence had no *PIK3CA* mutations. Generally, almost all patients except for those in case 18 and 32 did not have the same *PIK3CA* mutations at all sites. These results revealed the presence of intratumor heterogeneity of *PIK3CA* mutations in EMPD.

Discussion

We examined the frequency of *PIK3CA* mutations related to intertumor heterogeneity and intratumor heterogeneity, as well as the correlation between *PIK3CA* mutation patterns and clinical parameters. The frequency of one or more *PIK3CA* mutations was 30.8%, and the frequency of E542K, E545K, and H1047R was approximately 10%. These *PIK3CA* mutations rarely occur simultaneously and at approximately the same rate in EMPD, although the frequency of hotspot *PIK3CA* mutations differs in other cancers, including colon and breast cancers¹¹. There was no significant correlation between *PIK3CA* hotspot mutations and clinical parameters. Multiple *PIK3CA* mutations accelerate downstream PI3K-AKT signaling *in vitro*¹⁴. However, MMs of *PIK3CA* in EMPD were only present in 4.4% (3/68) of patients, and MMs of *PIK3CA* had no clinical

significance in our cohort. Further accumulation of patients with MMs of *PIK3CA* may reveal the clinical significance of MMs because there were three patients with EMPD with *PIK3CA* MMs.

Cancer is composed of multiple populations with different mutations (so called intratumor heterogeneity), and mutations in cancer are divided into two groups: founder mutations and progressor mutations²². Founder mutations accumulate in the early phase of cancer evolution, and the ancestral clones accumulate progressor mutations and branch into a subclone population²². *PIK3CA* mutations are progressor mutations in many types of cancer²³. In our study, 87.5% of 16 patients with *PIK3CA* mutations (14/16) had intratumor heterogeneity of *PIK3CA* mutations, which indicates that *PIC3CA* mutations may not be founder mutations but progressor mutations in EMPD, as in other cancers. In addition, in case2, there was only one lesion with the E542K mutation in the first surgical specimen, and no mutation was detected in the surgical specimen on recurrence. This result suggests that recurrent lesions may not be derived from the site with the E542K mutation.

In clinical trials, cancer with *PIK3CA* mutations predicted a good response to PI3K inhibitors¹³. Our study revealed that the frequency of one or more *PIK3CA* mutations in EMPD is approximately 30%, and there is intratumor heterogeneity of

PIK3CA mutations. If the PI3K inhibitor is used in daily practice, its response rate may not be 30% in EMPD. Considering the intratumor heterogeneity of *PIK3CA* mutations in EMPD, we need to recognize the possibility that the outcome of *PIK3CA*-related investigations may be a false negative depending on the collection site.

There are several limitations to this study. We examined only hotspot *PIK3CA* mutations using ddPCR, and other *PIK3CA* mutations were not investigated. Most examined tissues did not include a sufficient number of specimens of metastatic and recurrent lesions. In this study, all samples were derived from macrodissected FFPE tissues because several genetic tests of tissue samples, including BRAF V600 mutation test, are performed using not laser-microdissected but macrodissected tissues in a clinical setting. As a pilot study using 10 cases, we compared the results between macrodissected and laser-microdissected tissues. The VAF of laser-microdissected tissues was approximately four to ten times higher than that of macrodissected tissues (Supplemental Figure 5). Therefore, the resulting VAF in macrodissected tissues may have a false negative.

In conclusion, our study revealed intertumor and intratumor heterogeneity of *PIK3CA* mutations in EMPD.

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Figure legends.

Figure 1.

Representative amplification of *PIK3CA* mutation (E542K) in EMPD tissues by droplet digital polymerase chain reaction.

Figure 2.

Representative case of intratumor heterogeneity of *PIK3CA* mutations in EMPD. The number on the picture of the tumor tissue matches the number in the table. +: positive, -: negative.

Supplementary Figure 1.

Agarose gel electrophoresis of genomic DNAs to assess the quality. Each fragment size of DNA was from 300 bp to 2000 bp.

Supplementary Figure 2.

Representative amplification of *PIK3CA* Wild Type Reference standard (Horizon Discovery) as negative control by droplet digital polymerase chain reaction to assess the possibility of false positive rate for *PIK3CA* E545K.

Supplementary Figure 3.

Representative amplification of *PIK3CA* mutations in EMPD tissues by droplet digital polymerase chain reaction. (b) E545K, (c) H1047R.

Supplementary Figure 4.

All cases of intratumor heterogeneity of *PIK3CA* mutations in EMPD. The number on the picture of the tumor tissue matches the number in the table. +: positive, -: negative.

Supplementary Figure 5.

Representative amplification of *PIK3CA* E542K in EMPD tissues derived from macrodissected and laser-microdissected FFPE tissues by droplet digital polymerase chain reaction.

Supplementary Figure 6.

Kaplan–Meier curves for 5-year survival of patients with EMPD categorized according to *PIK3CA* mutations.

Table 1.

M; male, F; female. *PIK3CA* mutations (E542K, E545K, and H1047R) and clinical manifestations (age, sex, degree of invasion, lymph node metastasis, distant metastasis) in 68 patients with EMPD.

Table 2.

SD; standard deviation. Correlations between *PIK3CA* mutations and clinical parameters.

Figure1

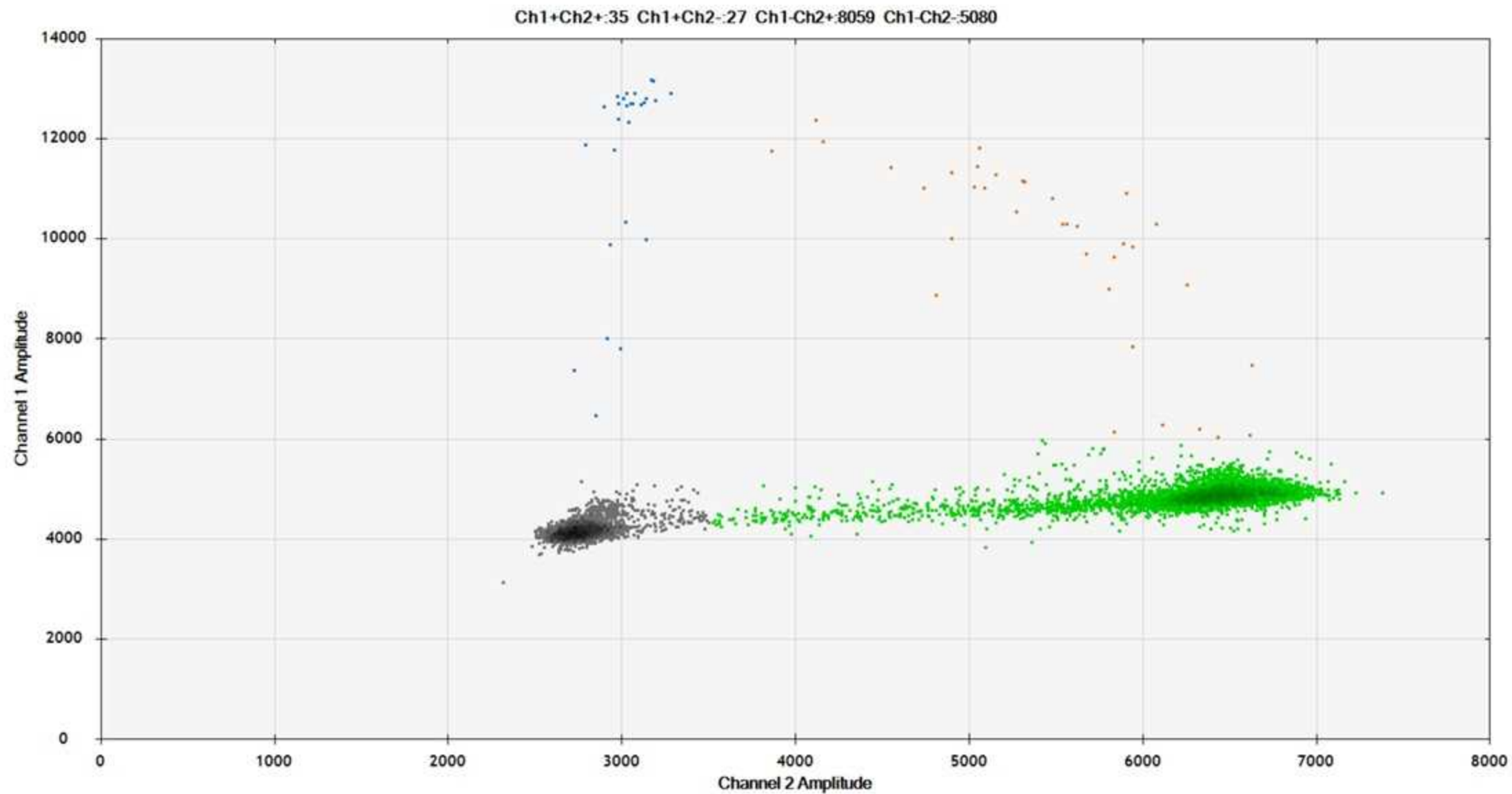
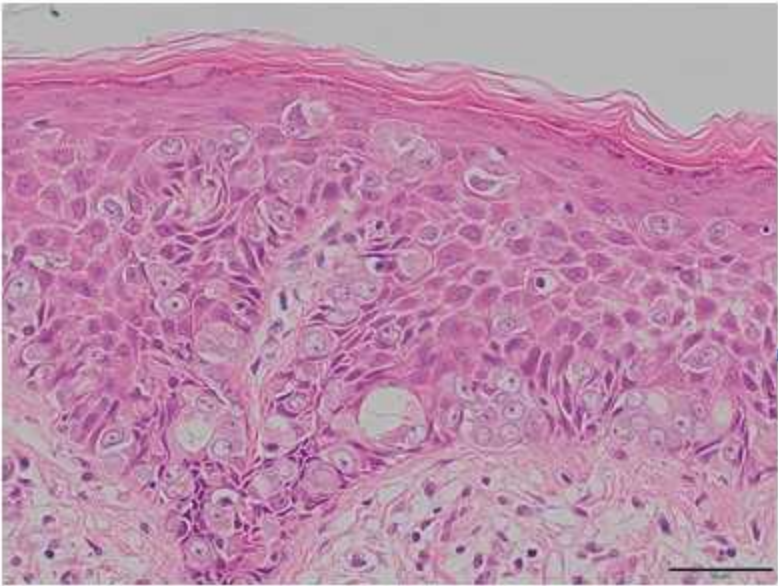
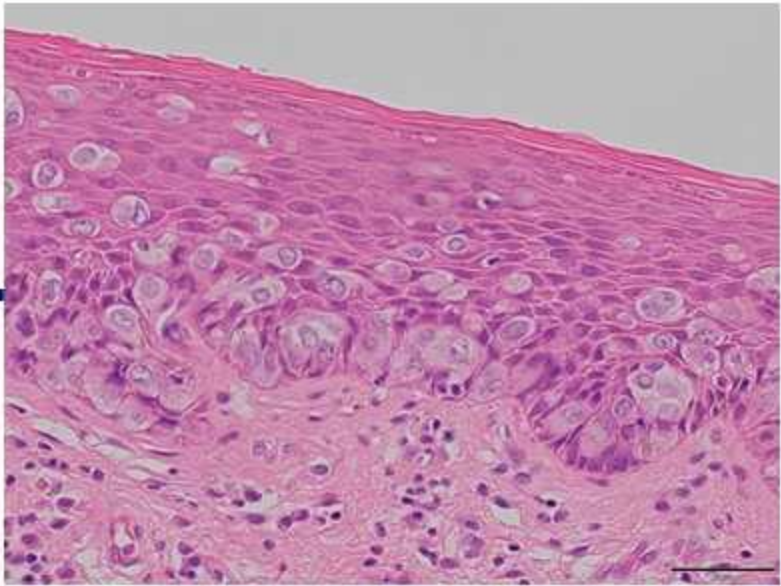
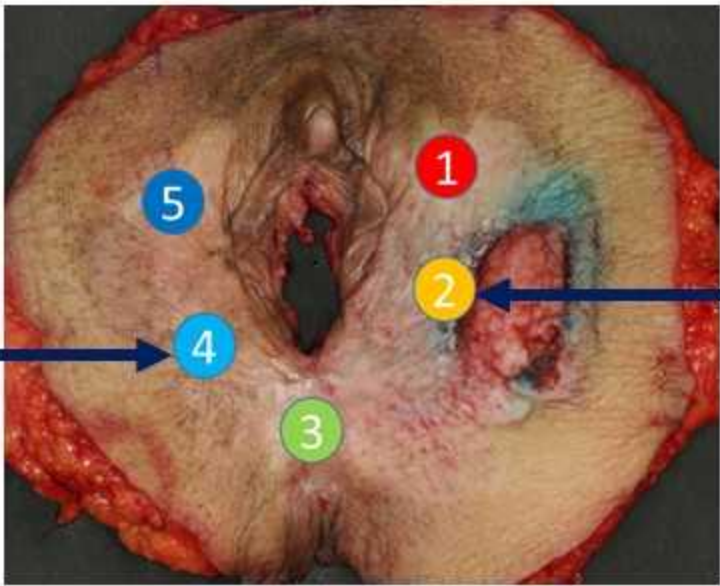


Figure2



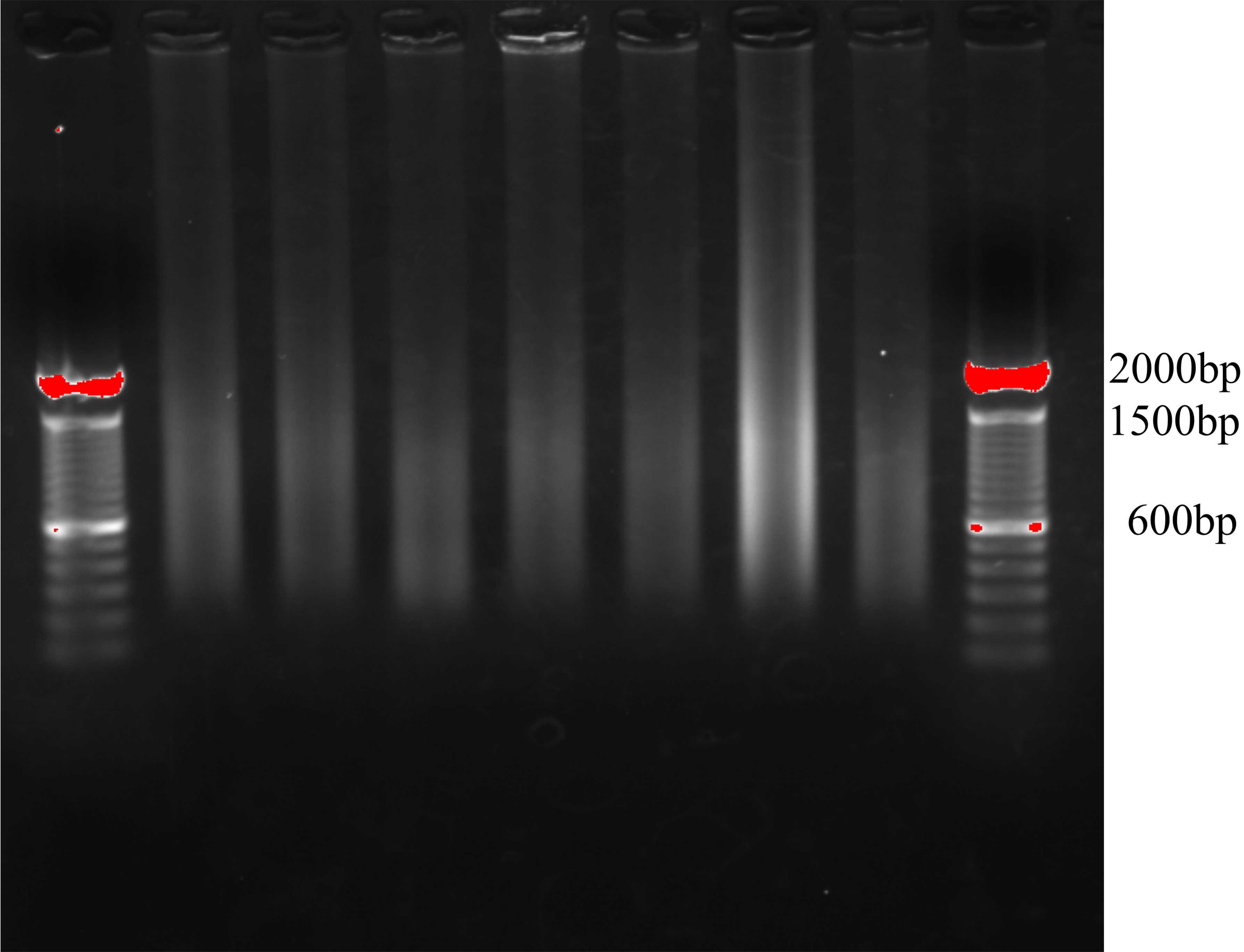
PIK3CA mutation – negative area



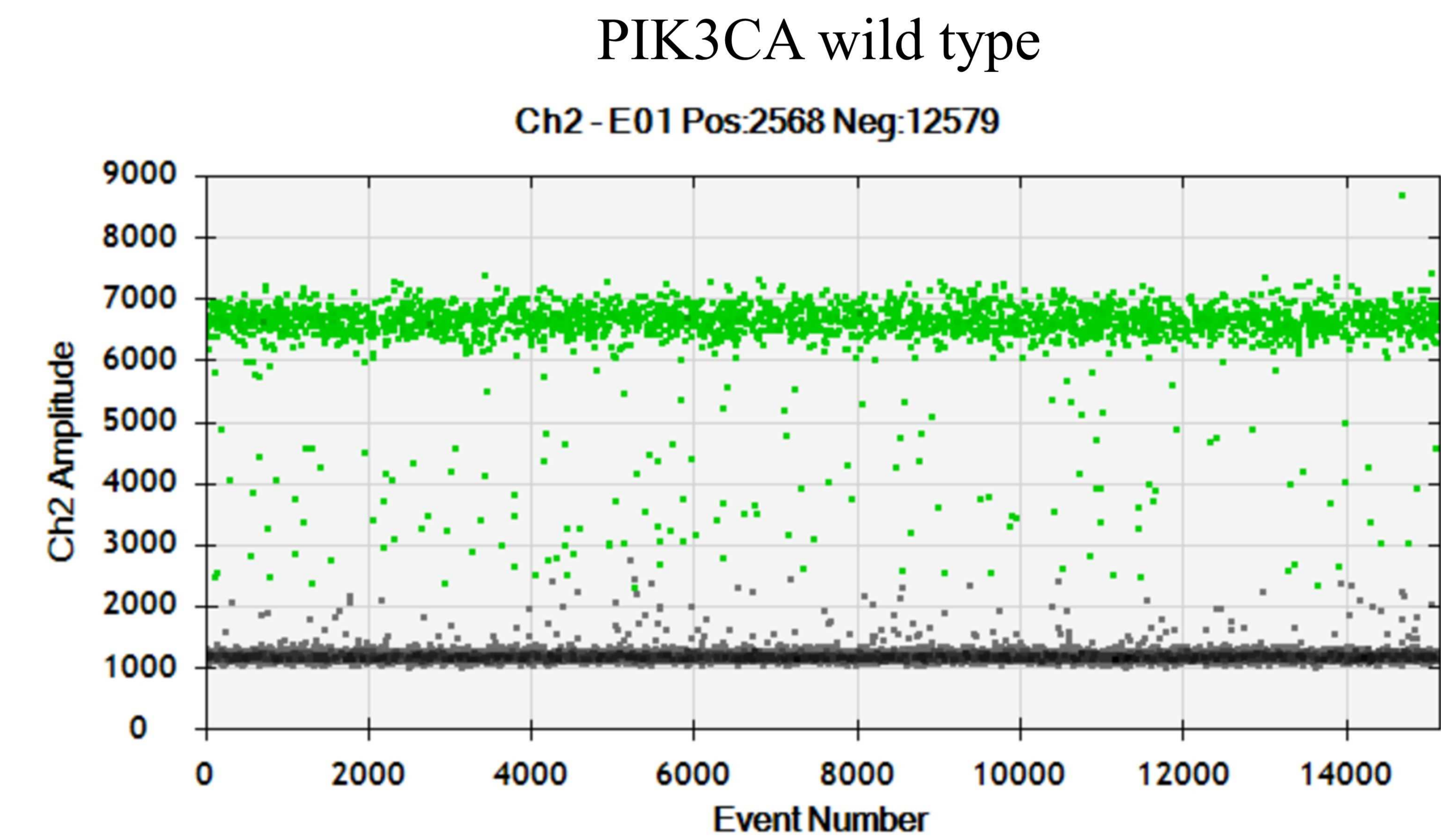
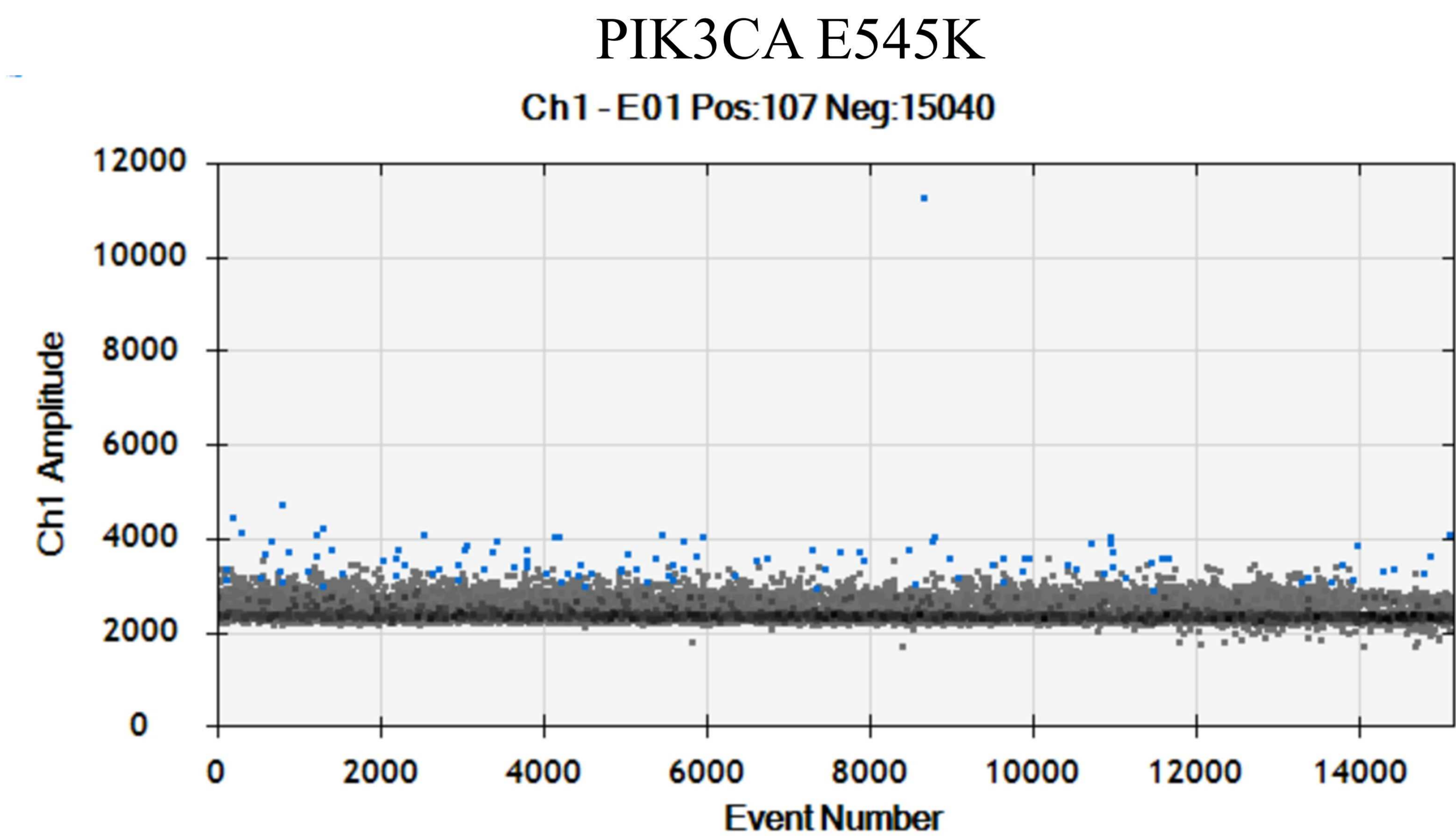
PIK3CA mutation – positive area

Mutation	①	②	③	④	⑤
E542K	-	+	-	-	-
E545K	-	-	-	-	-
H1047R	-	-	-	-	-

Supplemental Figure1

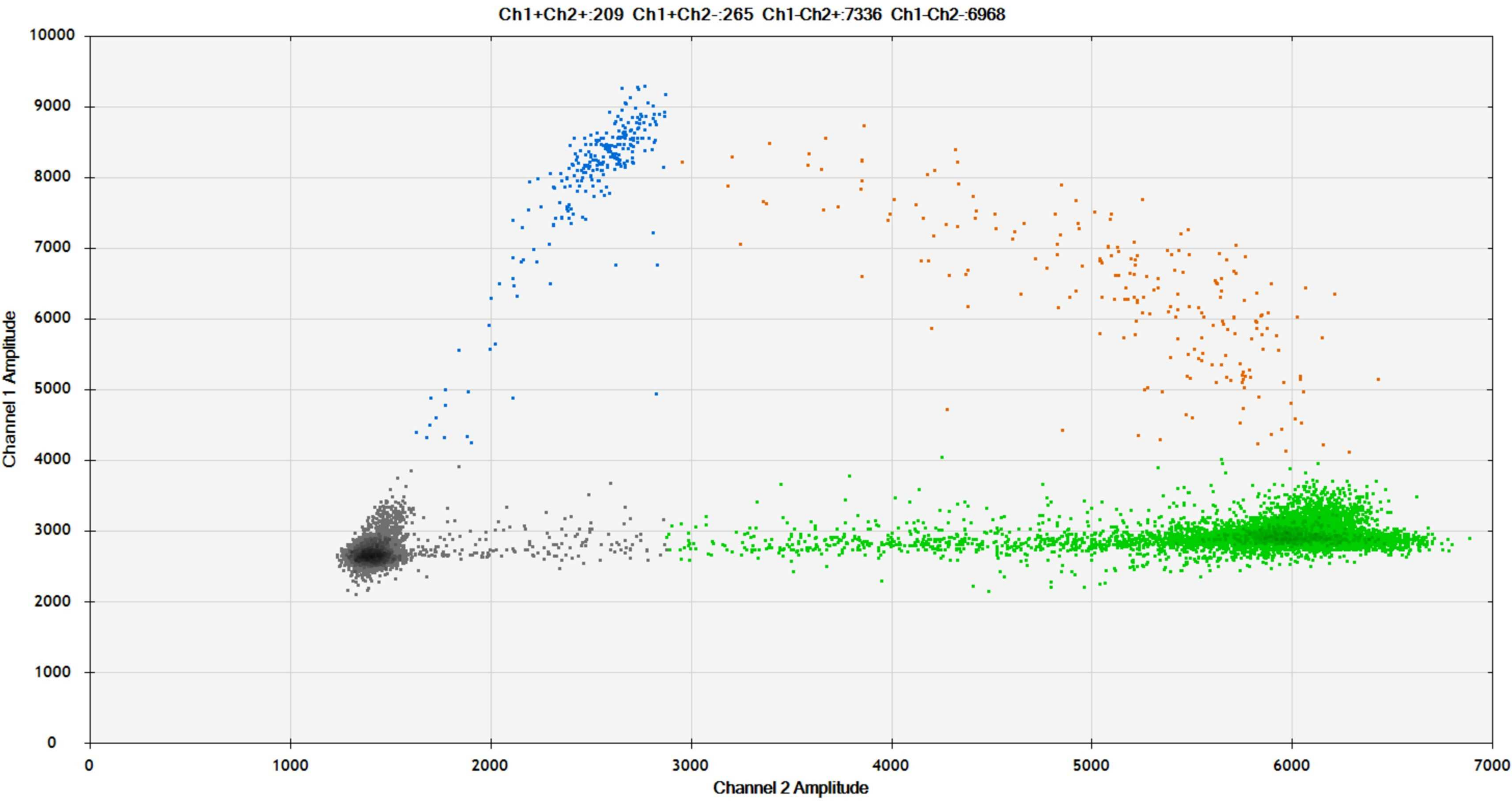


Supplemental Figure 2

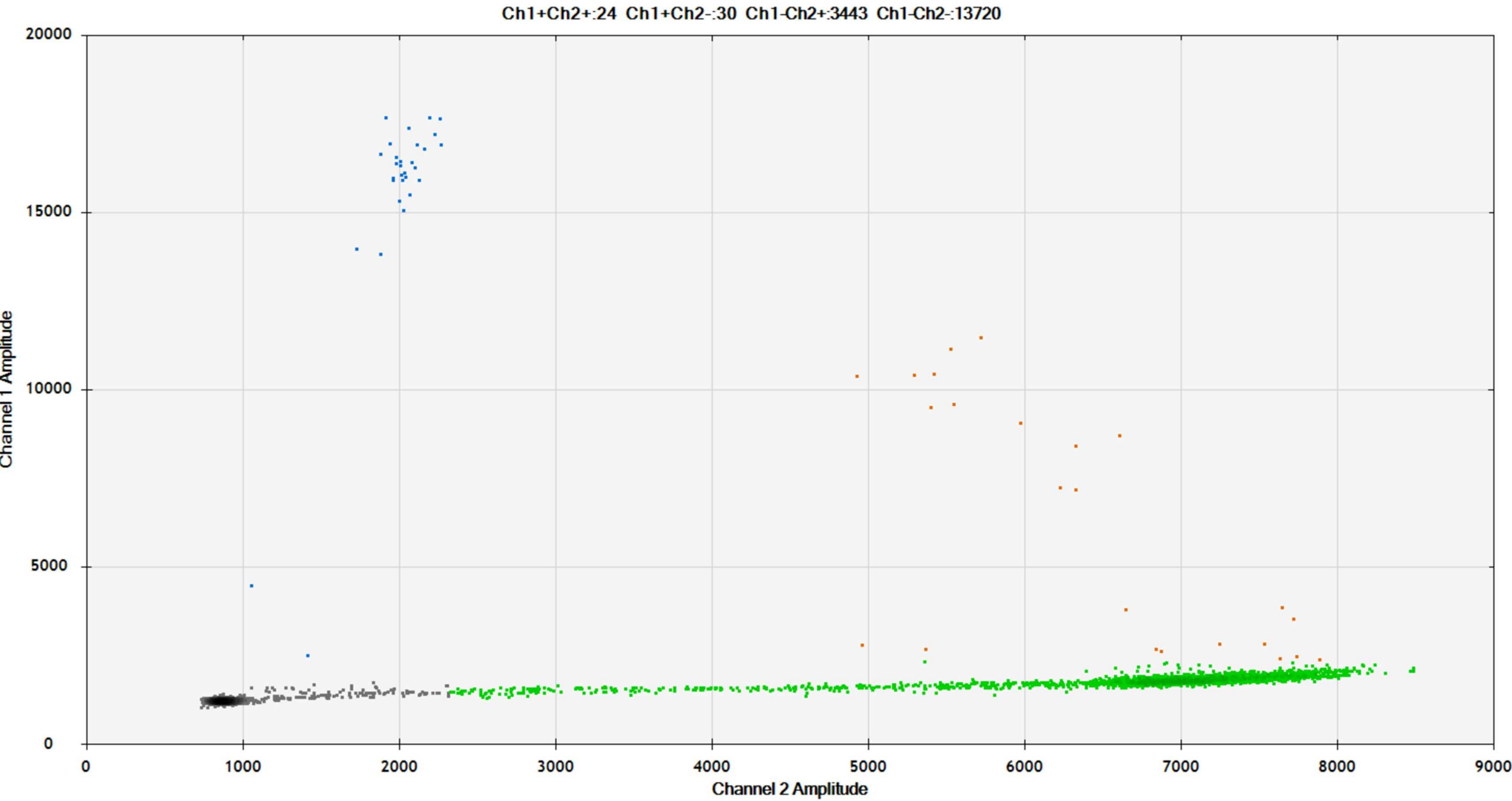


Supplemental Figure 3

(a)

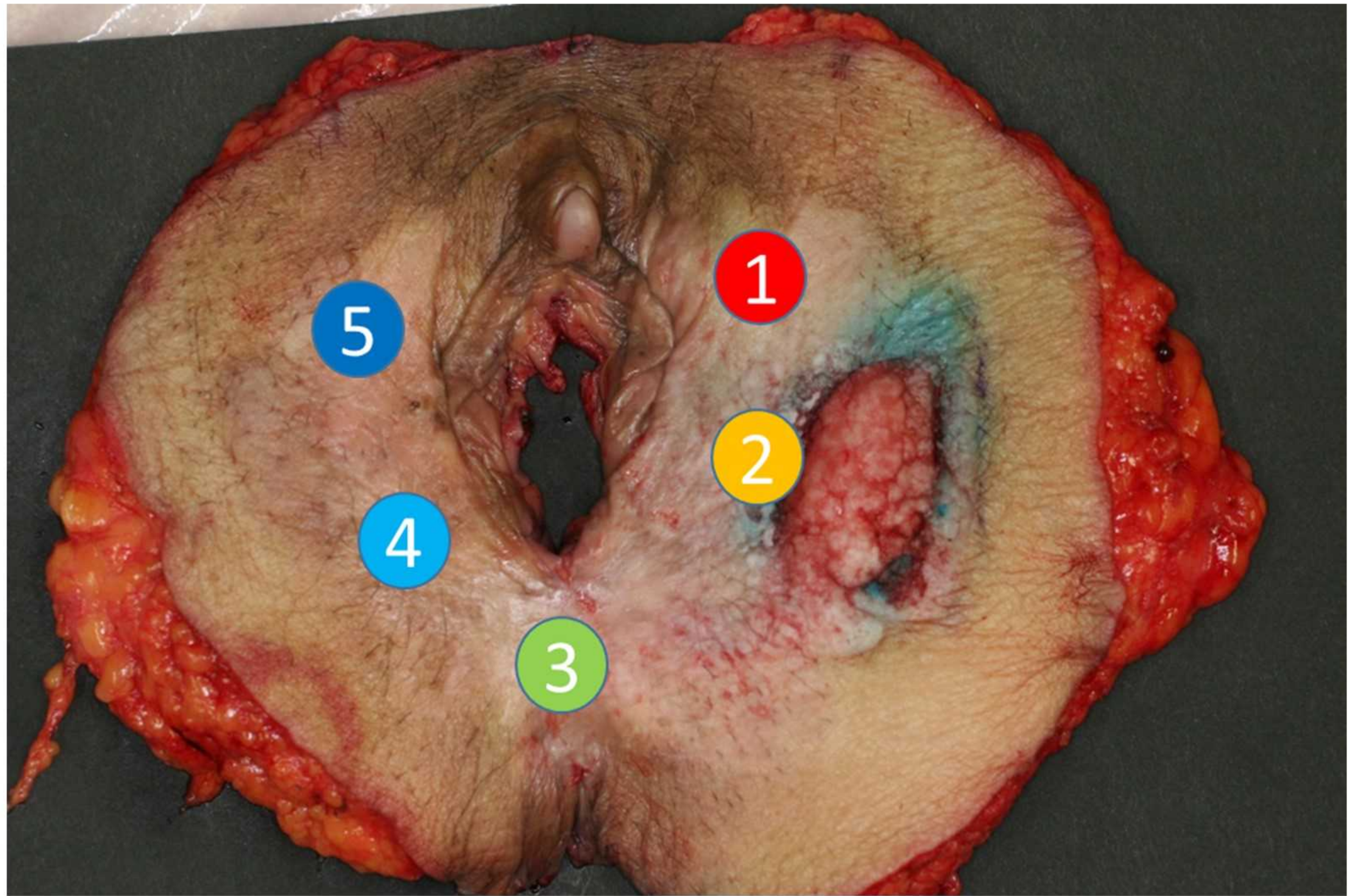


(b)



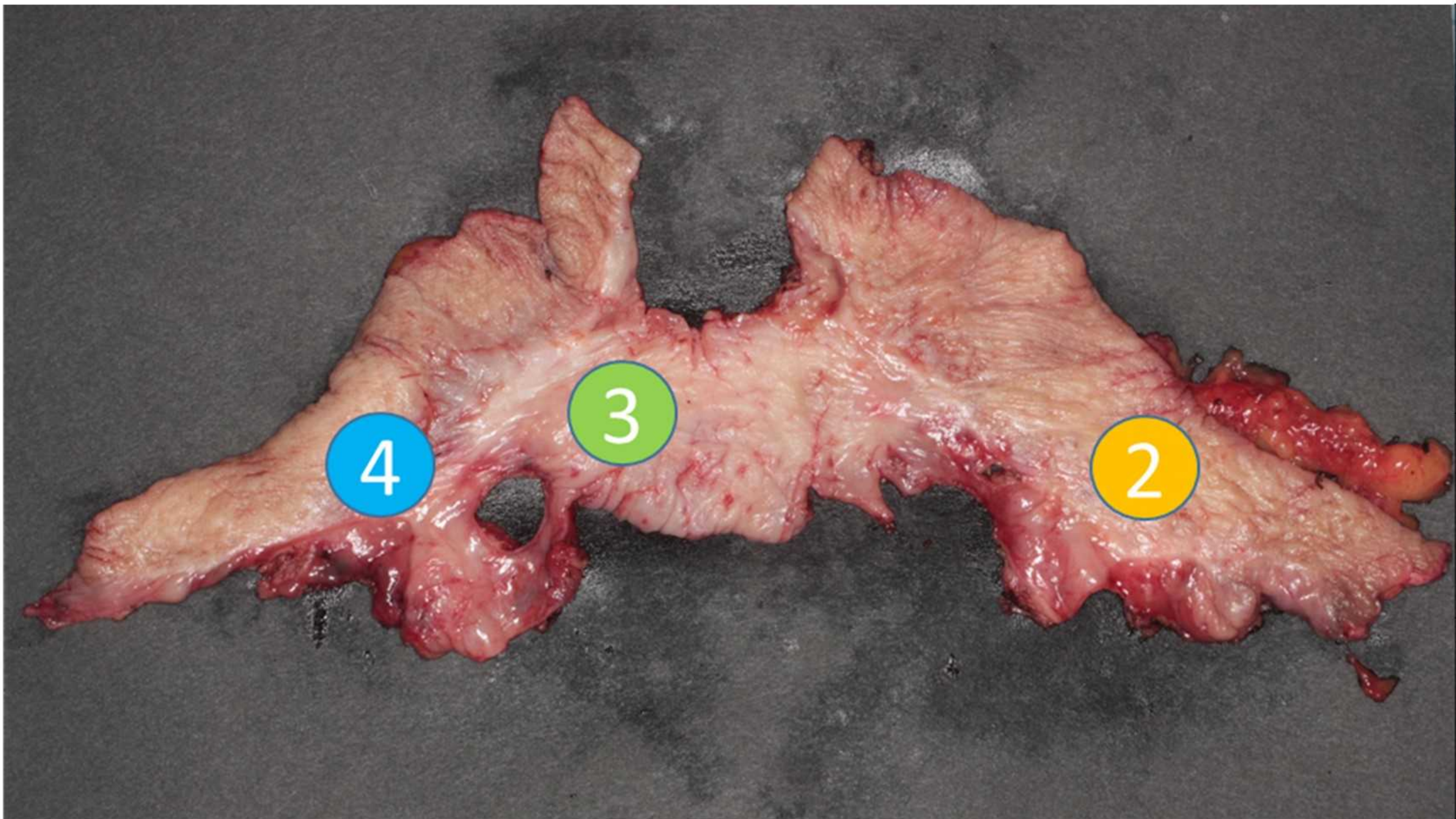
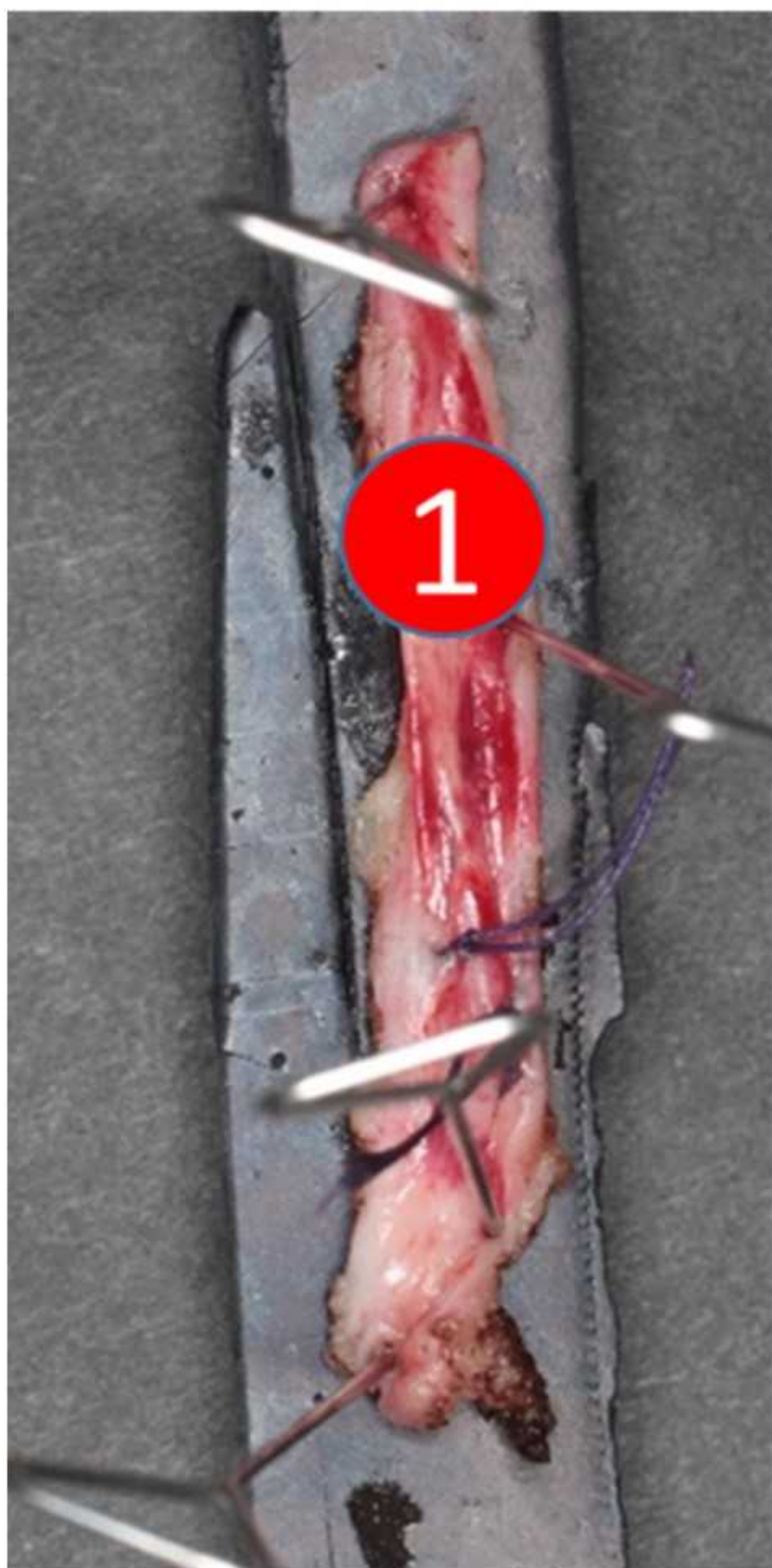
Supplemental Figure 4

Case2 first surgery



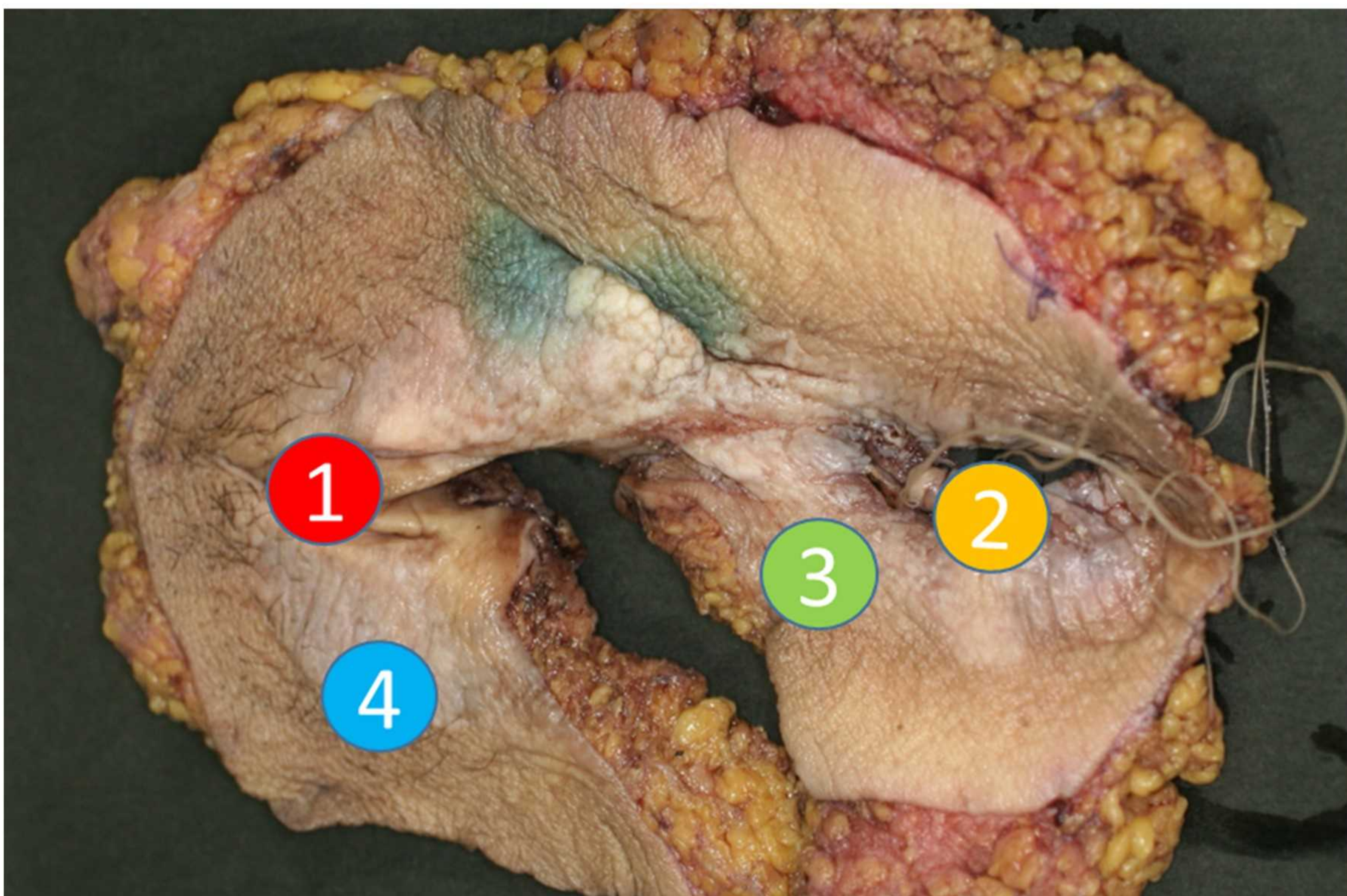
Mutation	①	②	③	④	⑤
E542K	—	+	-	-	-
E545K	-	-	-	-	-
H1047R	-	-	-	-	-

Case2 surgery on recurrence



Mutation	①	②	③	④
E542K	-	-	-	-
E545K	-	-	-	-
H1047R	-	-	-	-

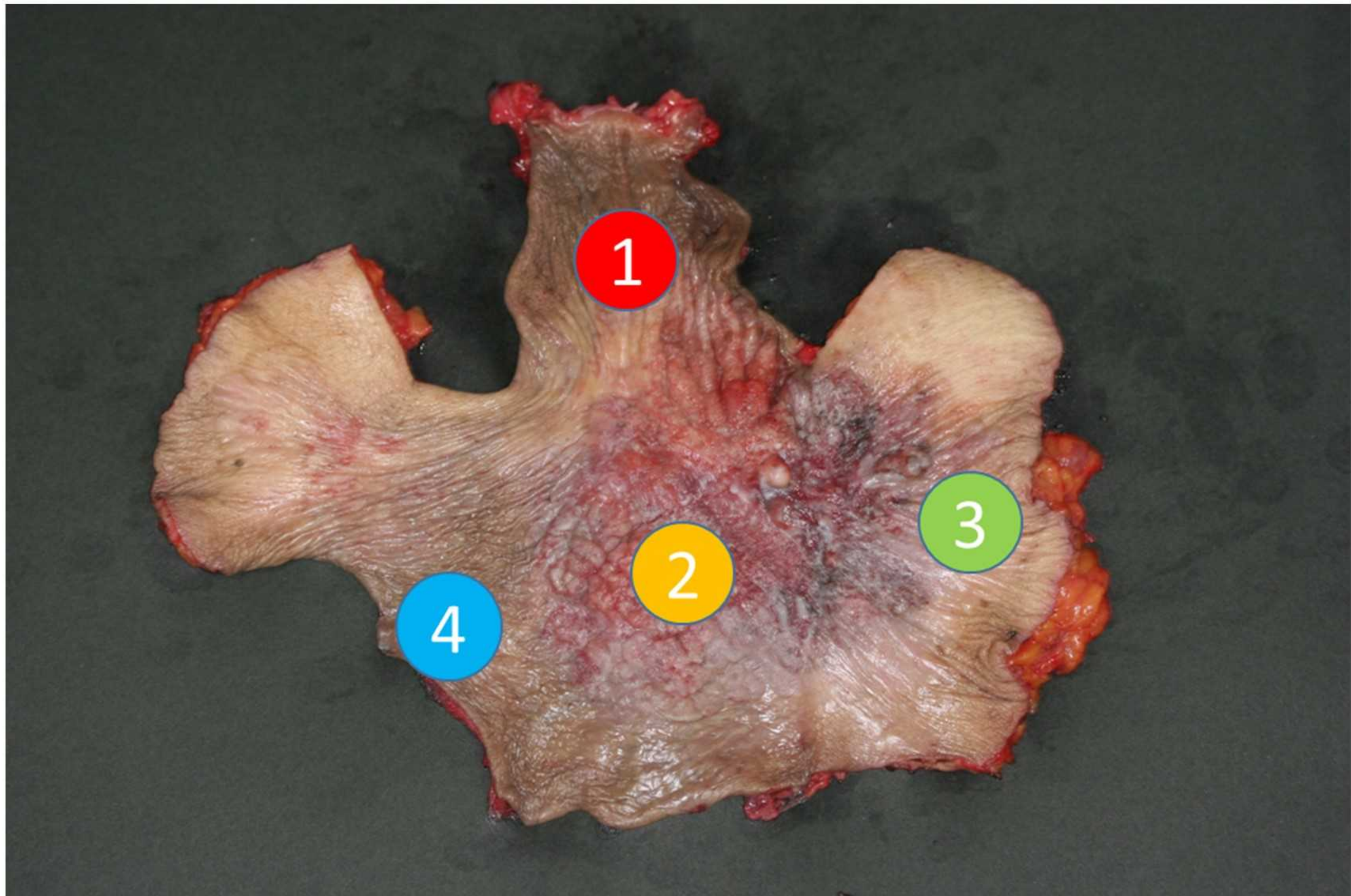
Case3



Mutation	①	②	③	④	⑤
E542K	+	-	-	-	-
E545K	-	+	-	-	-
H1047R	-	+	-	-	-

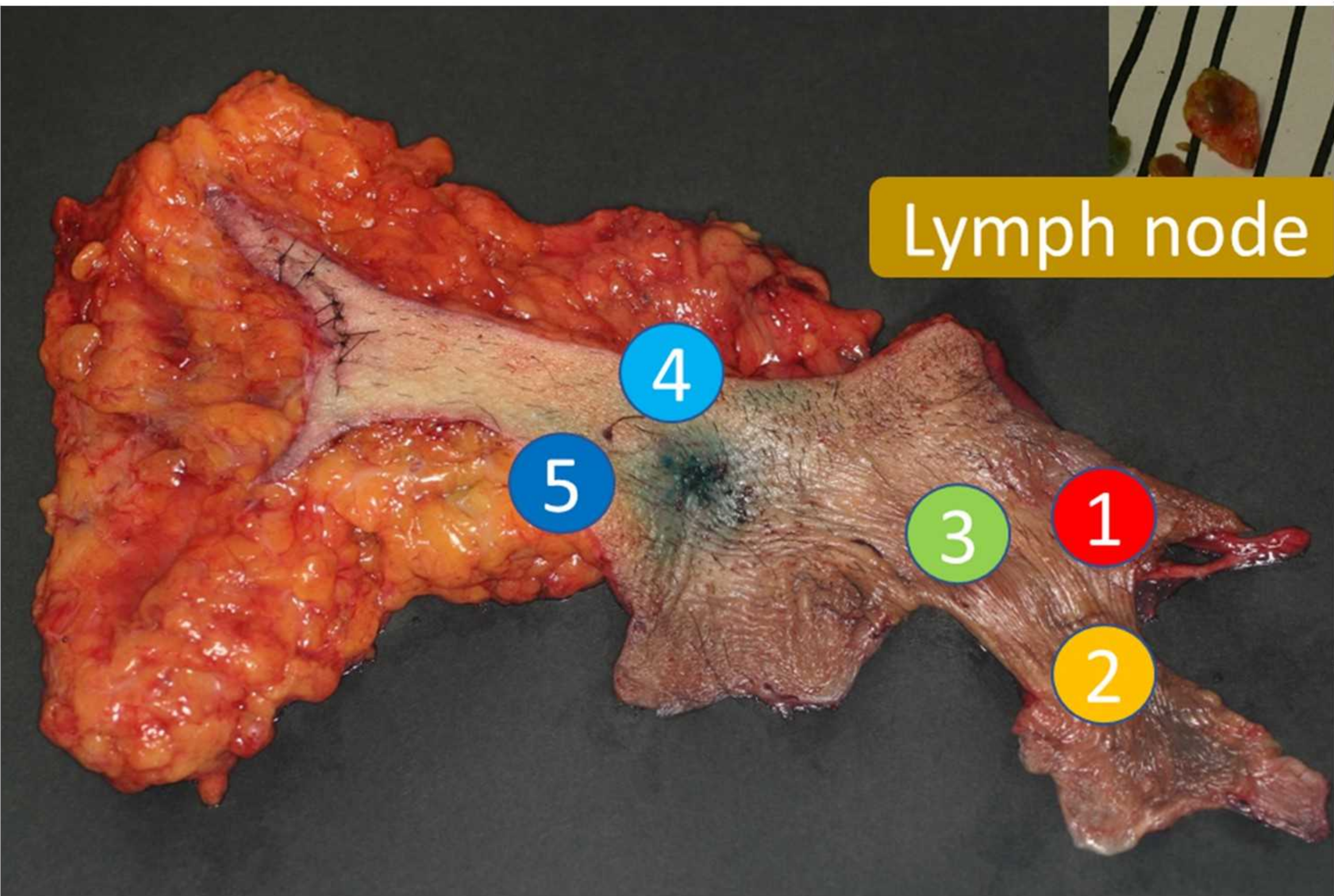
Supplemental Figure 4

Case8



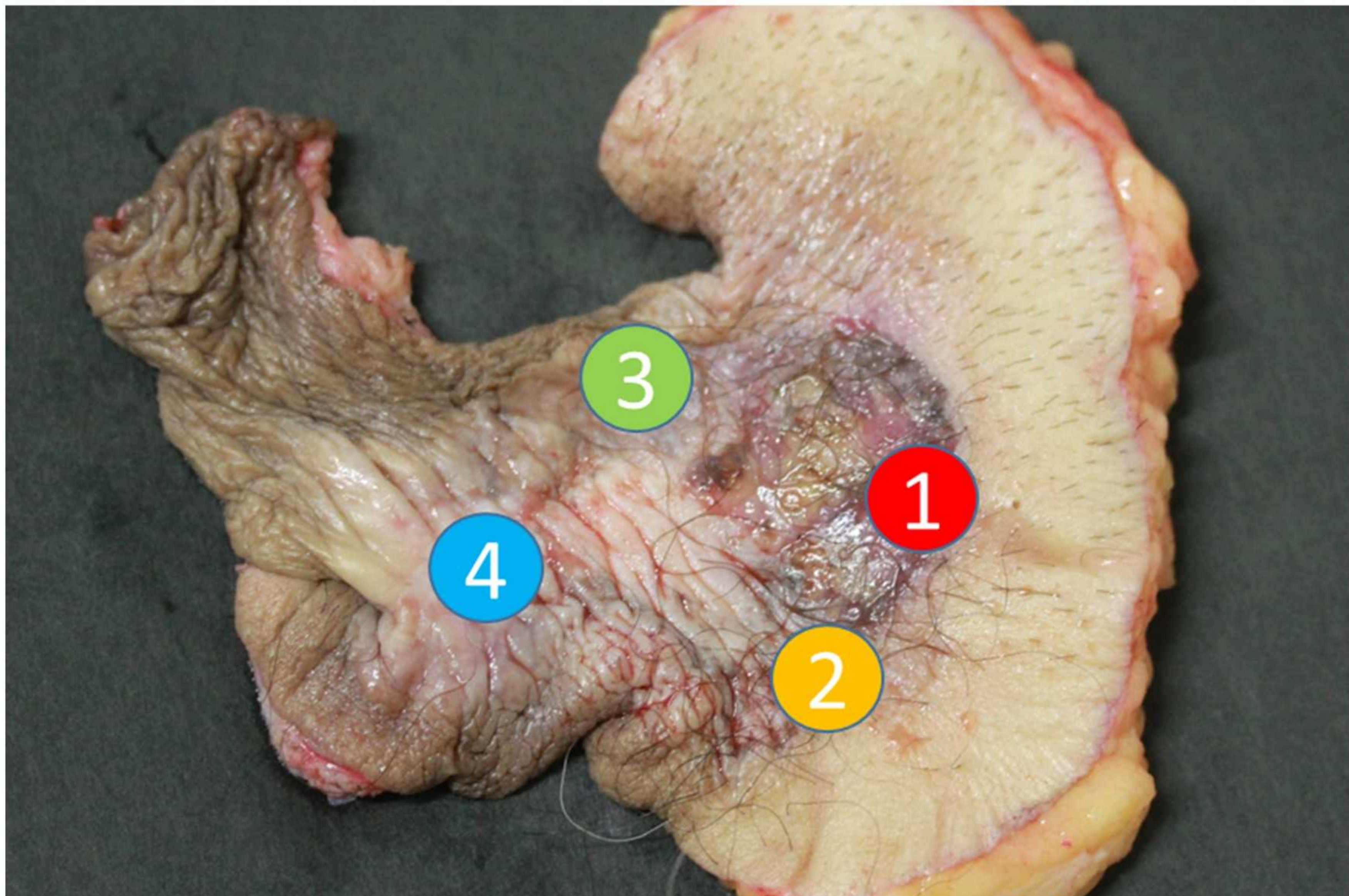
Mutation	①	②	③	④
E542K	-	-	-	-
E545K	-	-	-	-
H1047R	+	-	-	-

Case9



Mutation	①	②	③	④	⑤	Lymphnode metastasis
E542K	+	+	-	-	+	-
E545K	+	-	-	-	-	-
H1047R	-	-	-	-	-	-

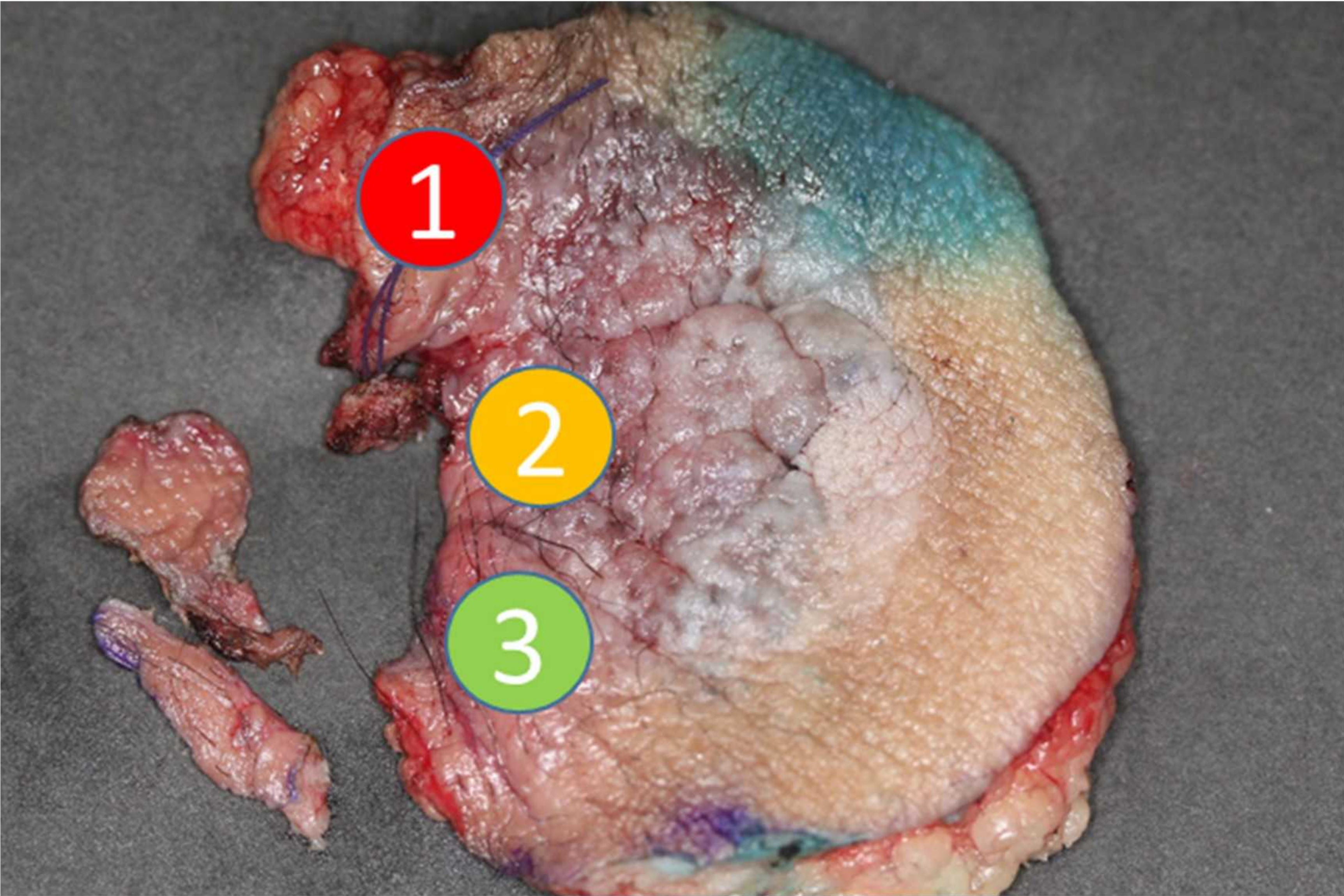
Case17



Mutation	①	②	③	④
E542K	-	+	-	-
E545K	+	-	-	-
H1047R	-	-	-	-

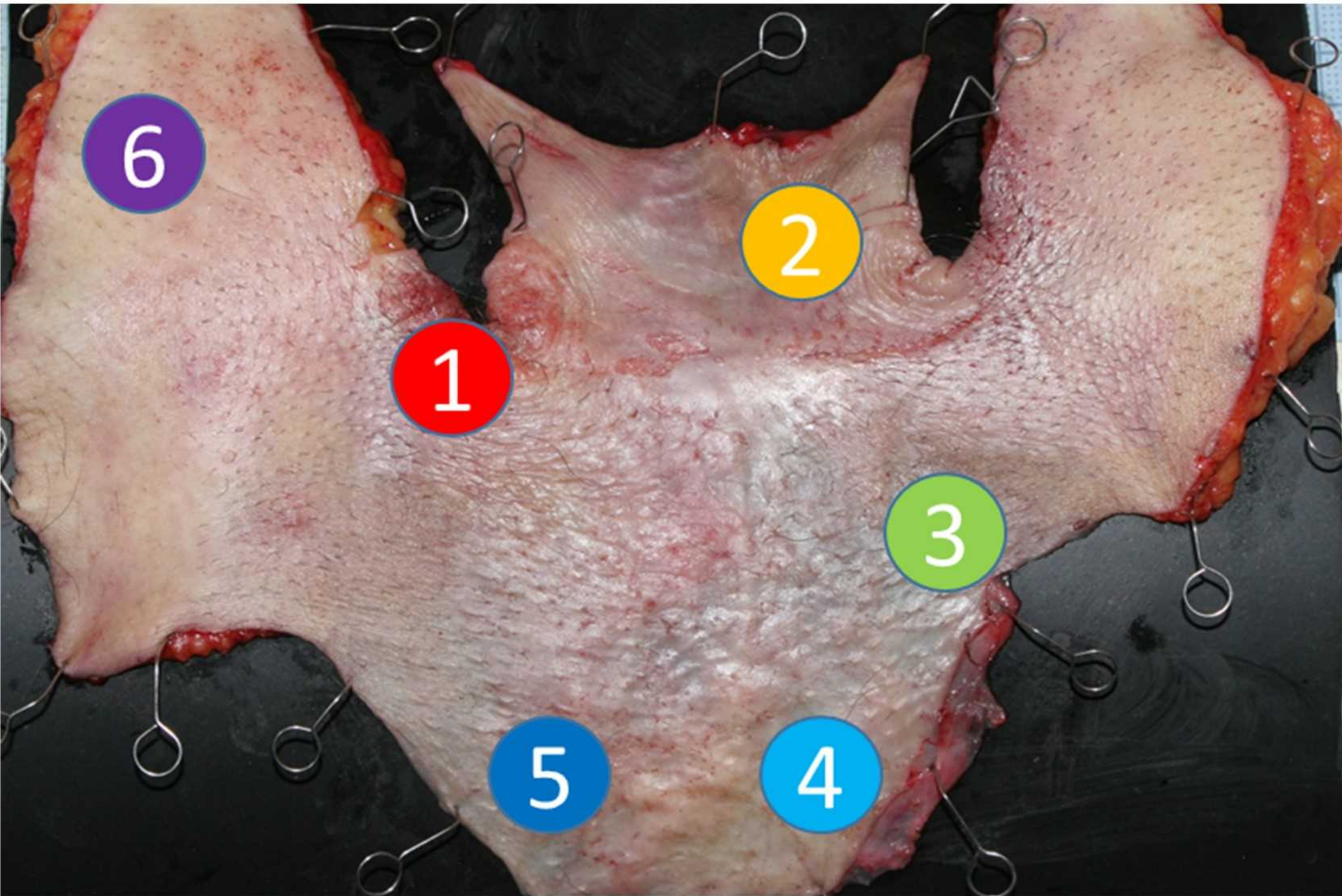
Supplemental Figure 4

Case18



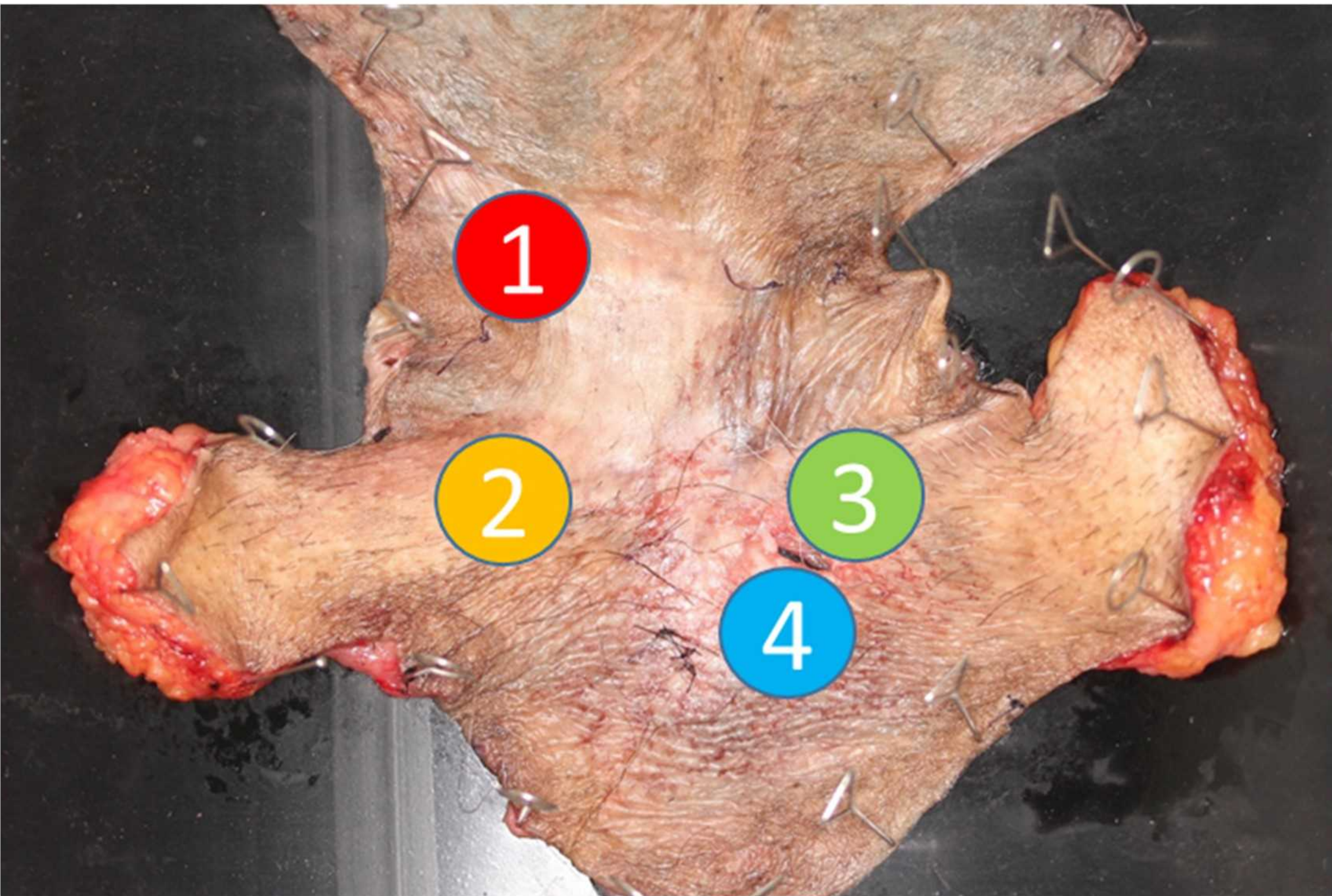
Mutation	①	②	③
E542K	-	-	-
E545K	+	+	+
H1047R	-	-	-

Case19



Mutation	①	②	③	④	⑤
E542K	-	-	-	+	-
E545K	-	-	-	-	-
H1047R	+	-	-	-	-

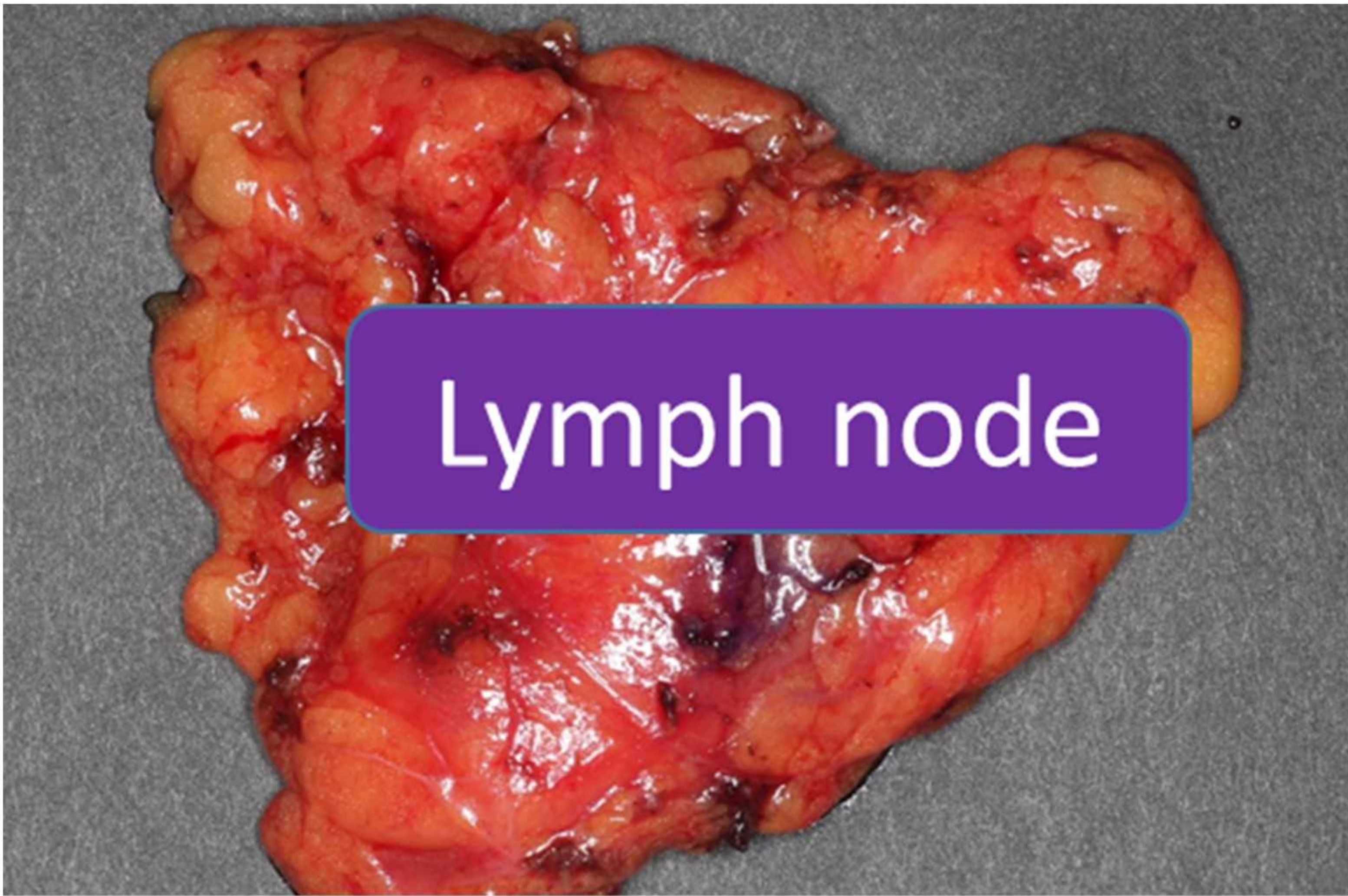
Case21



Mutation	①	②	③	④	⑤
E542K	+	-	-	-	-
E545K	+	-	-	-	-
H1047R	-	-	-	-	-

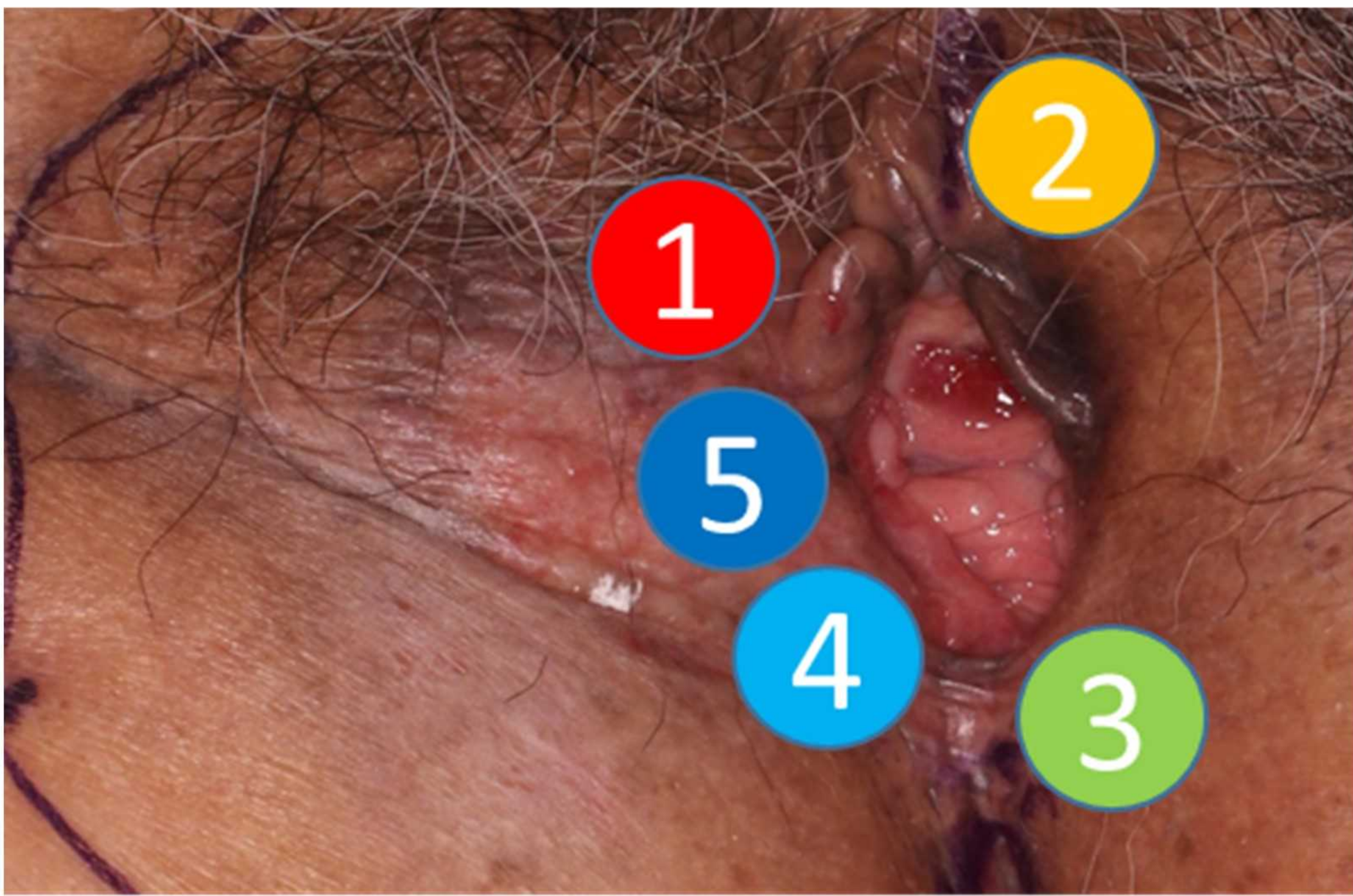
Supplemental Figure 4

Case32



Mutation	①	②	③	④	⑤	Lymphnode metastasis
E542K	+	+	+	+	+	+
E545K	-	-	-	-	-	-
H1047R	-	-	-	-	-	-

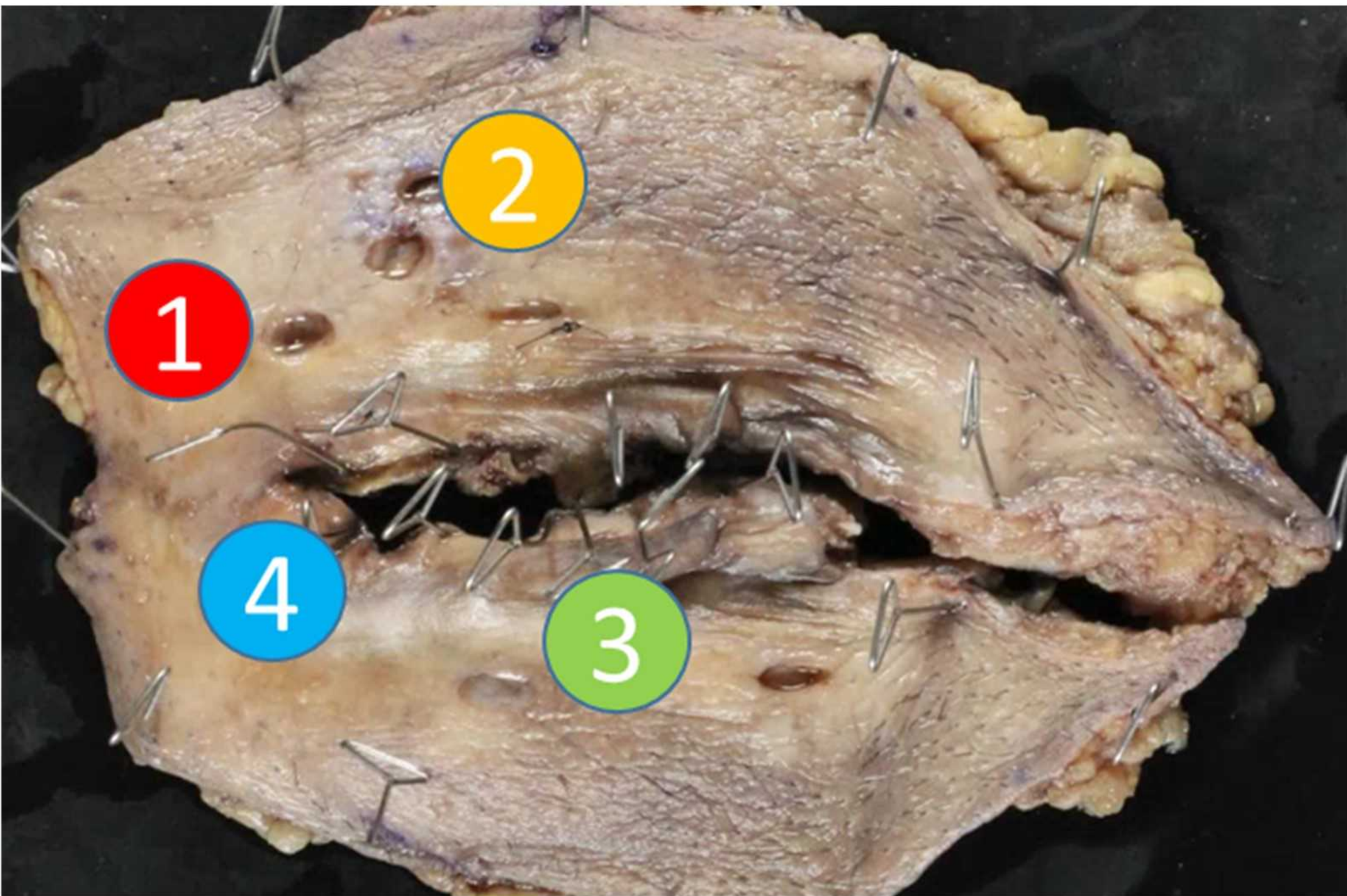
Case37



Mutation	①	②	③	④	⑤
E542K	+	-	-	+	-
E545K	-	-	-	-	-
H1047R	-	-	-	-	-

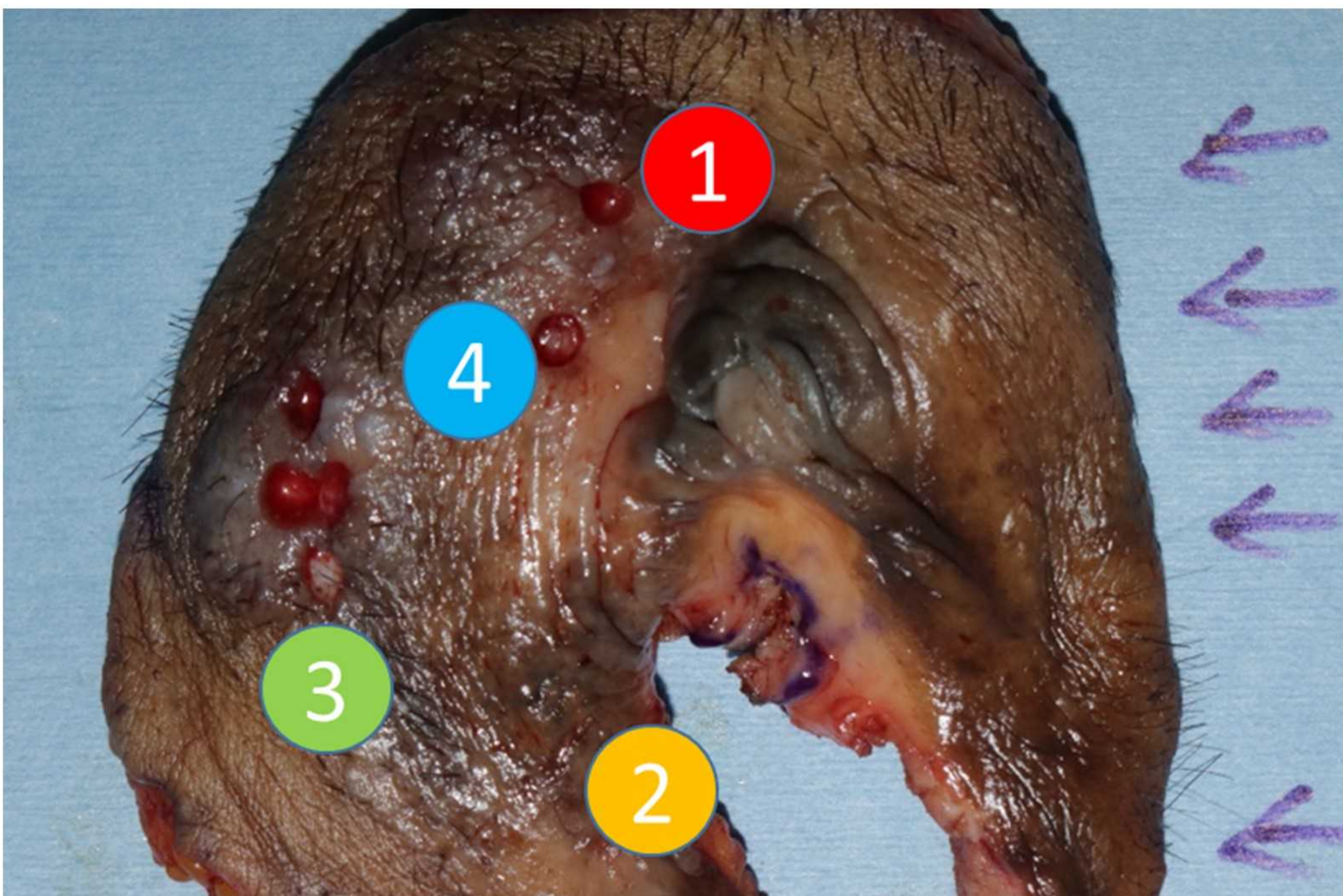
Supplemental Figure 4

Case41



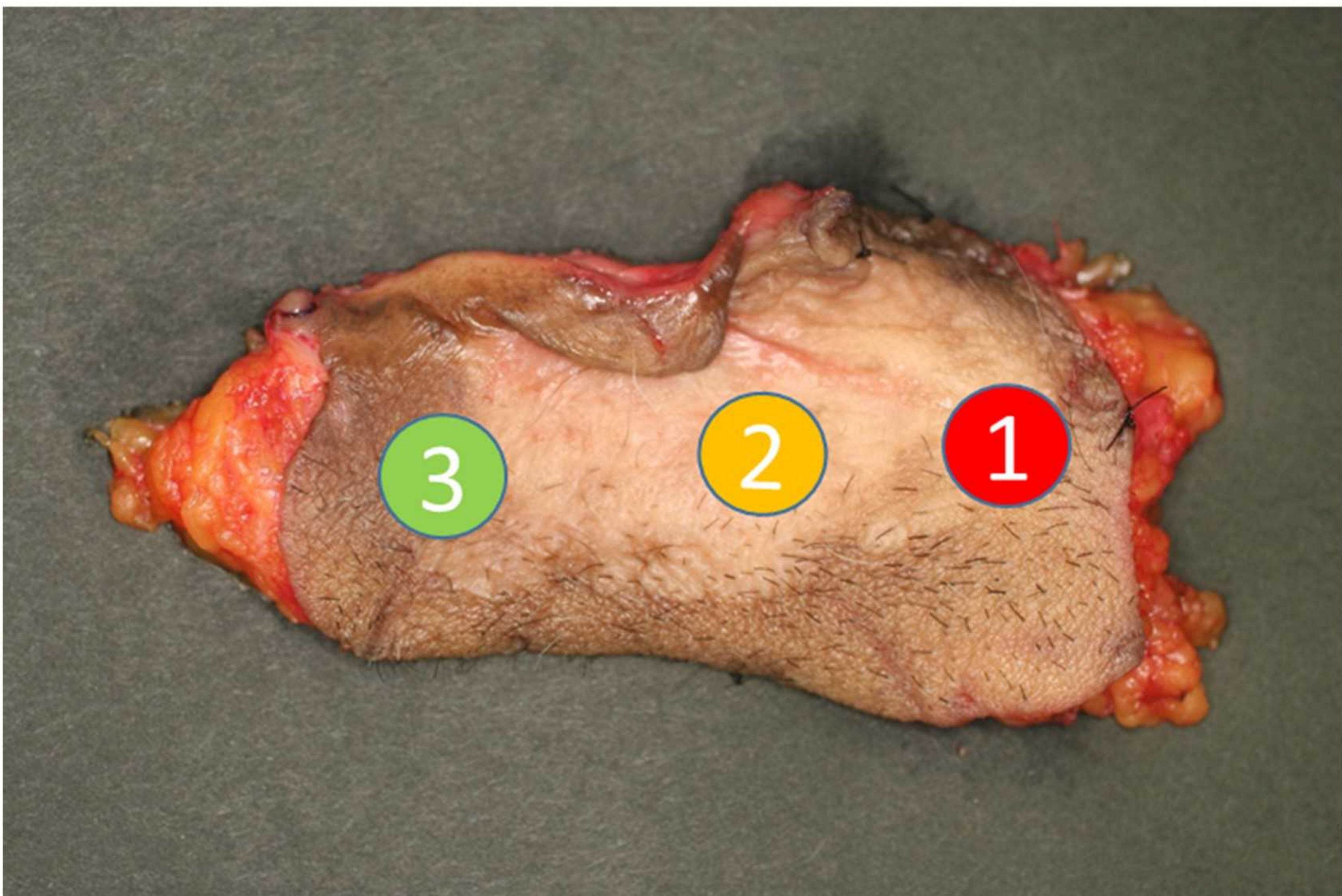
Mutation	①	②	③	④
E542K	-	-	-	-
E545K	-	-	-	-
H1047R	+	-	-	+

Case49



Mutation	①	②	③	④	⑤
E542K	-	-	-	-	-
E545K	-	-	-	-	-
H1047R	+	-	+	+	-

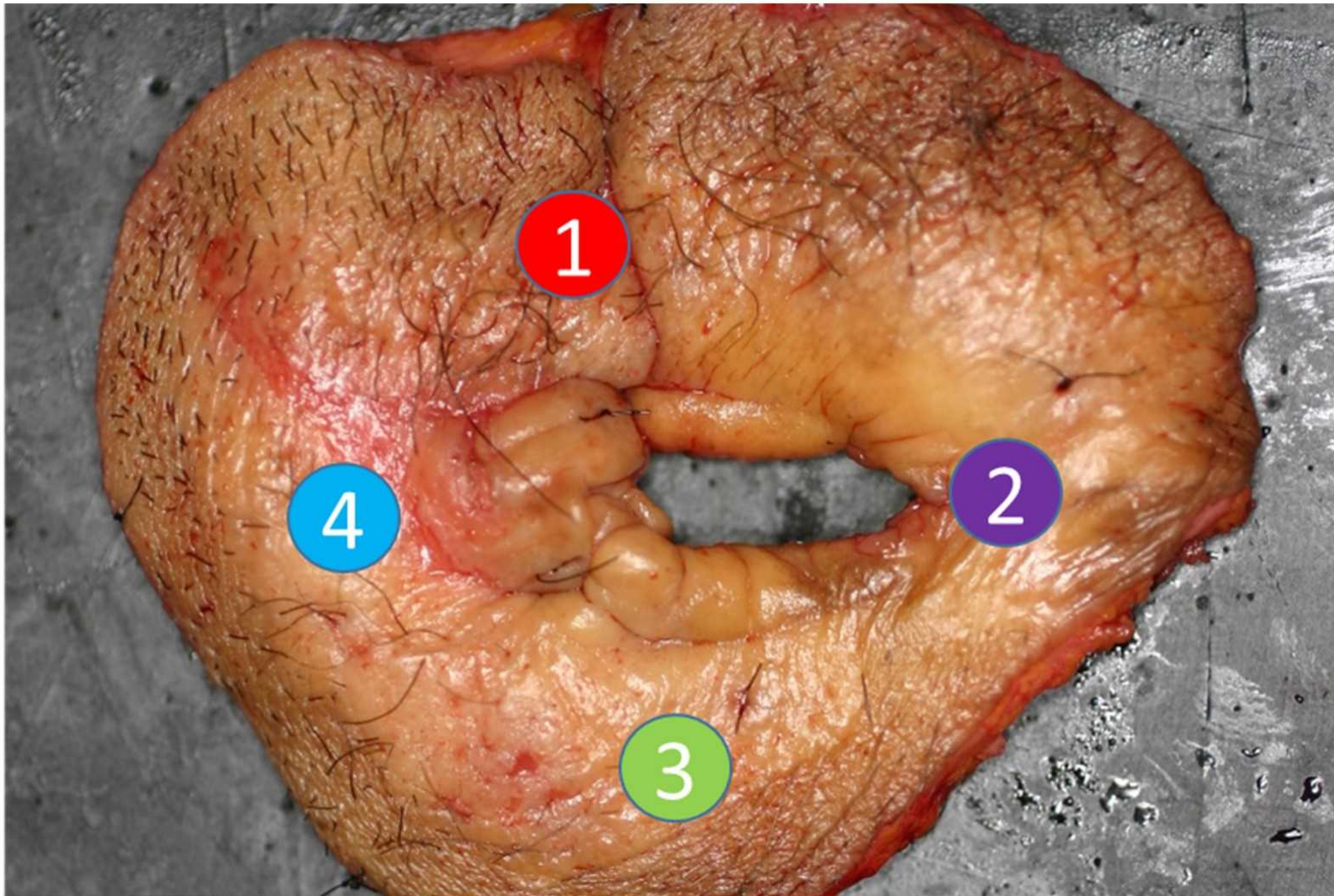
Case50



Mutation	①	②	③
E542K	+	-	-
E545K	-	-	-
H1047R	-	-	-

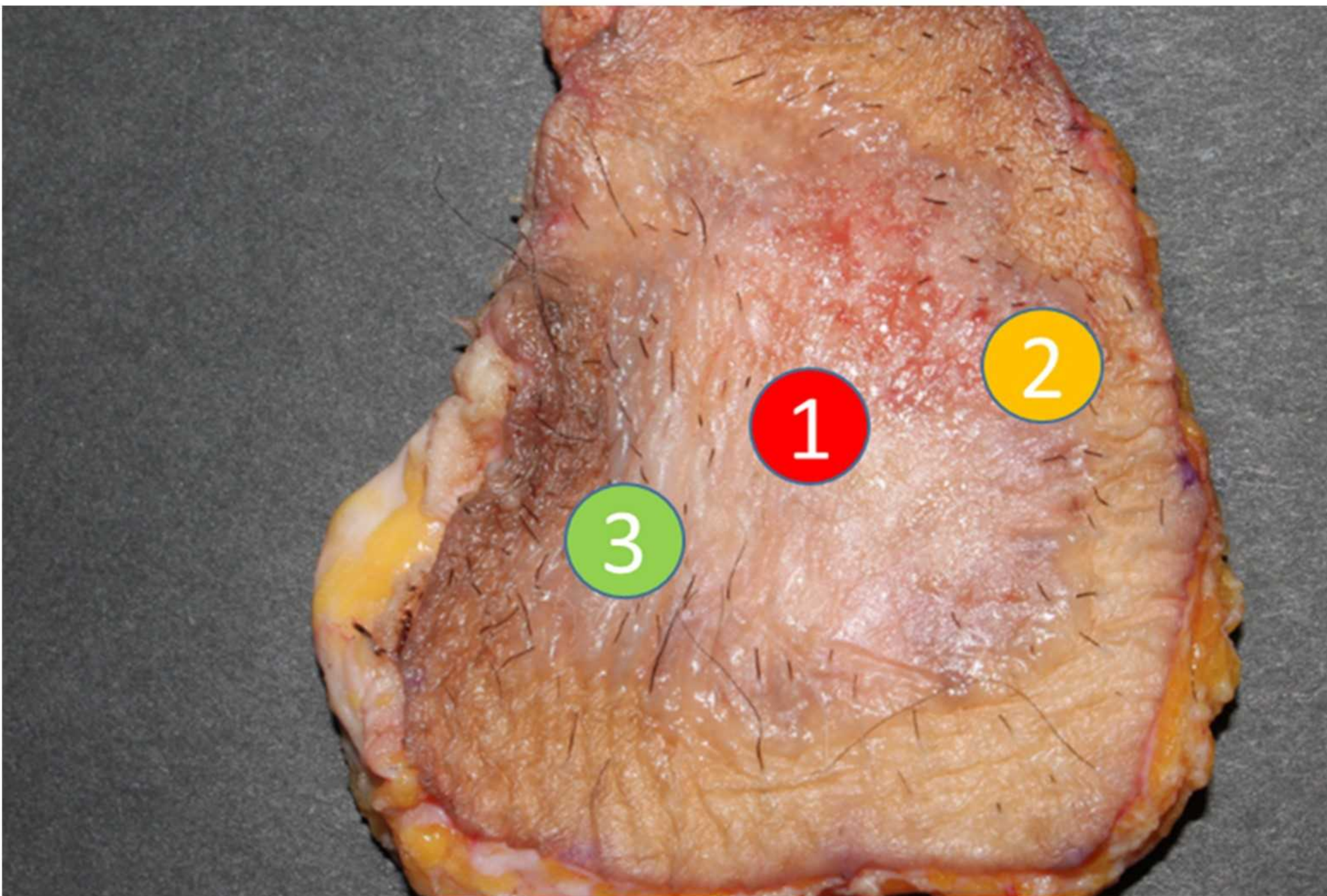
Supplemental Figure 4

Case53



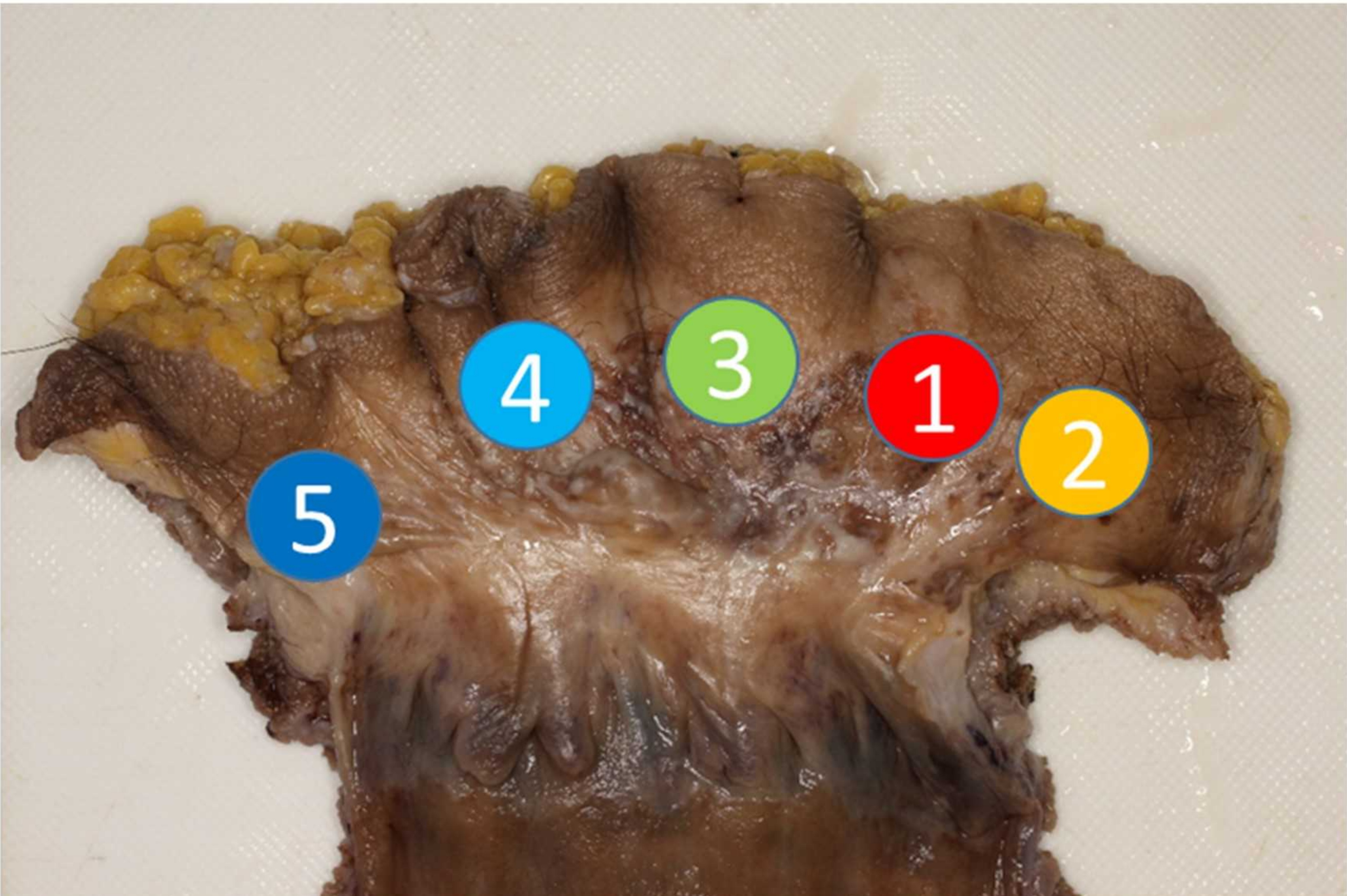
Mutation	①	②	③	④
E542K	-	-	-	-
E545K	+	-	+	-
H1047R	-	-	-	-

Case56



Mutation	①	②	③
E542K	-	-	-
E545K	+	-	-
H1047R	-	-	-

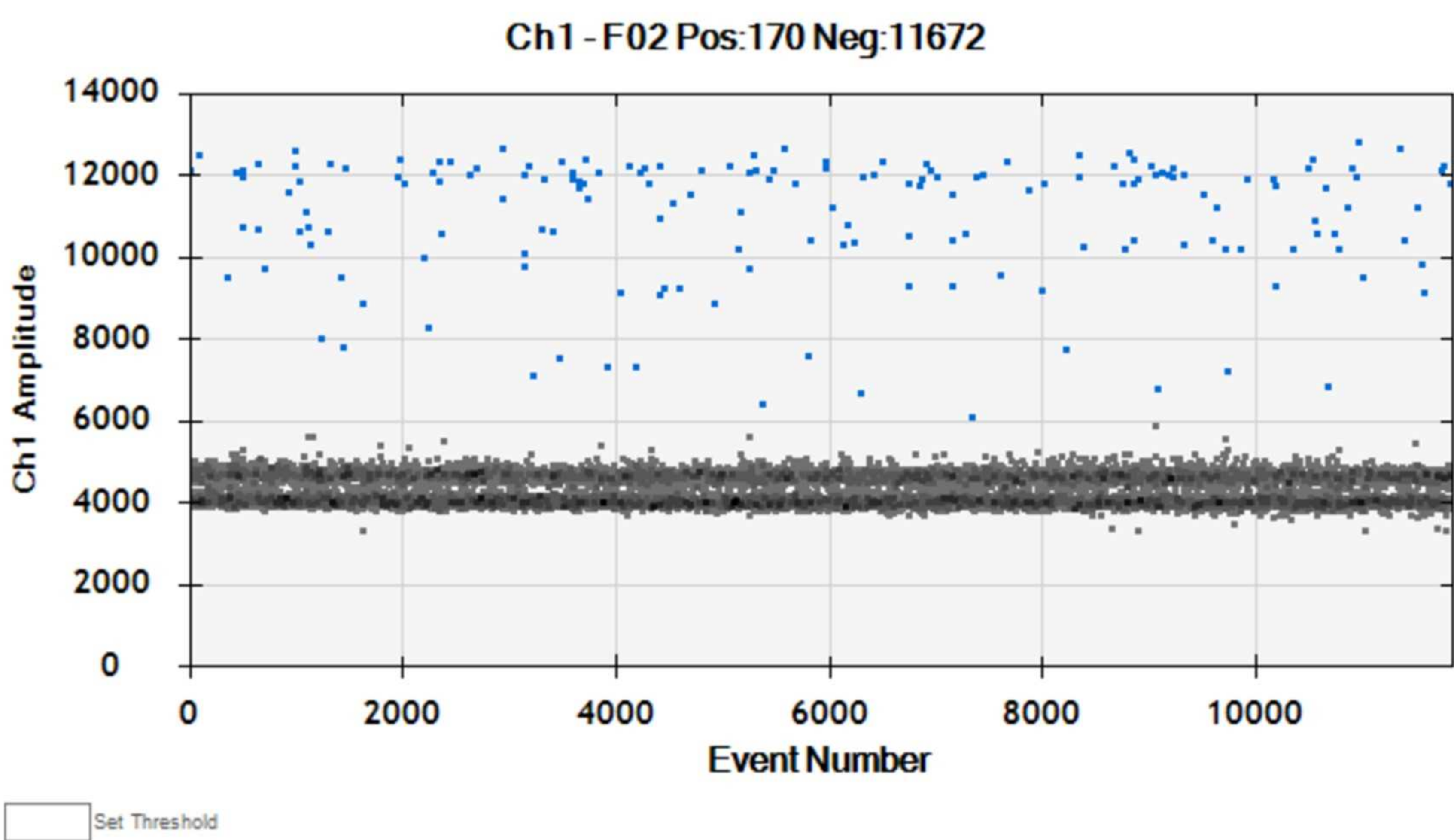
Case65



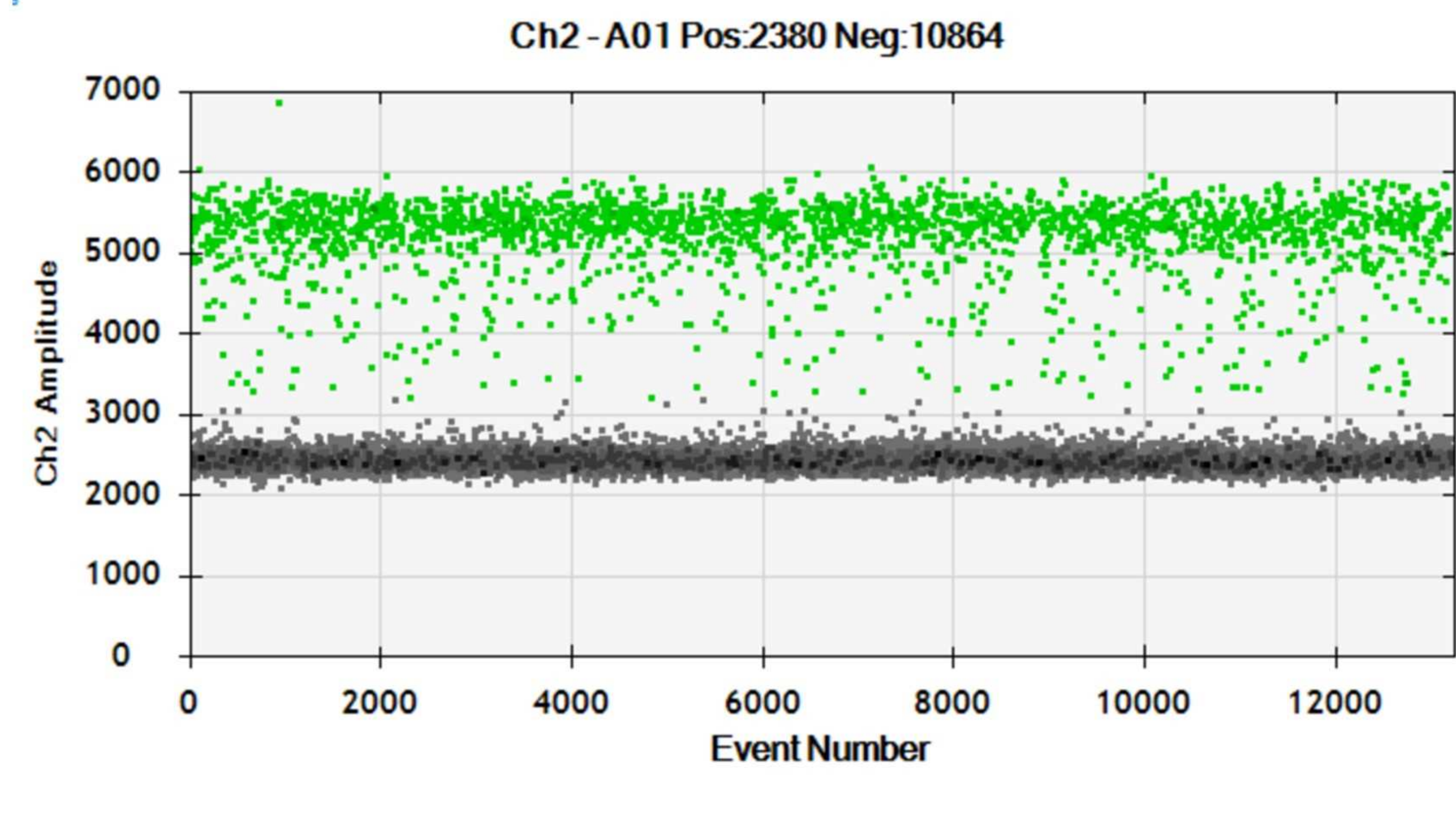
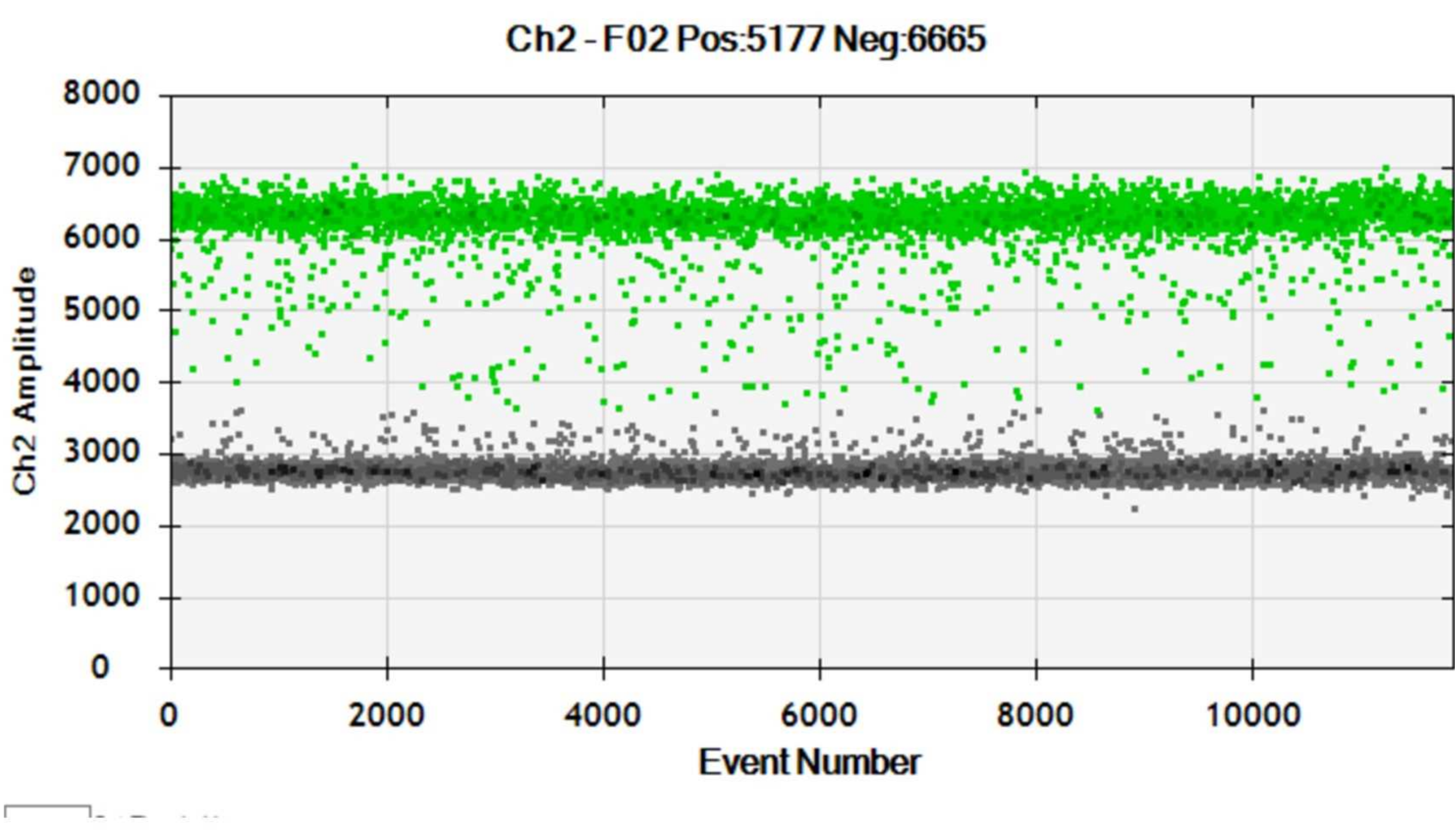
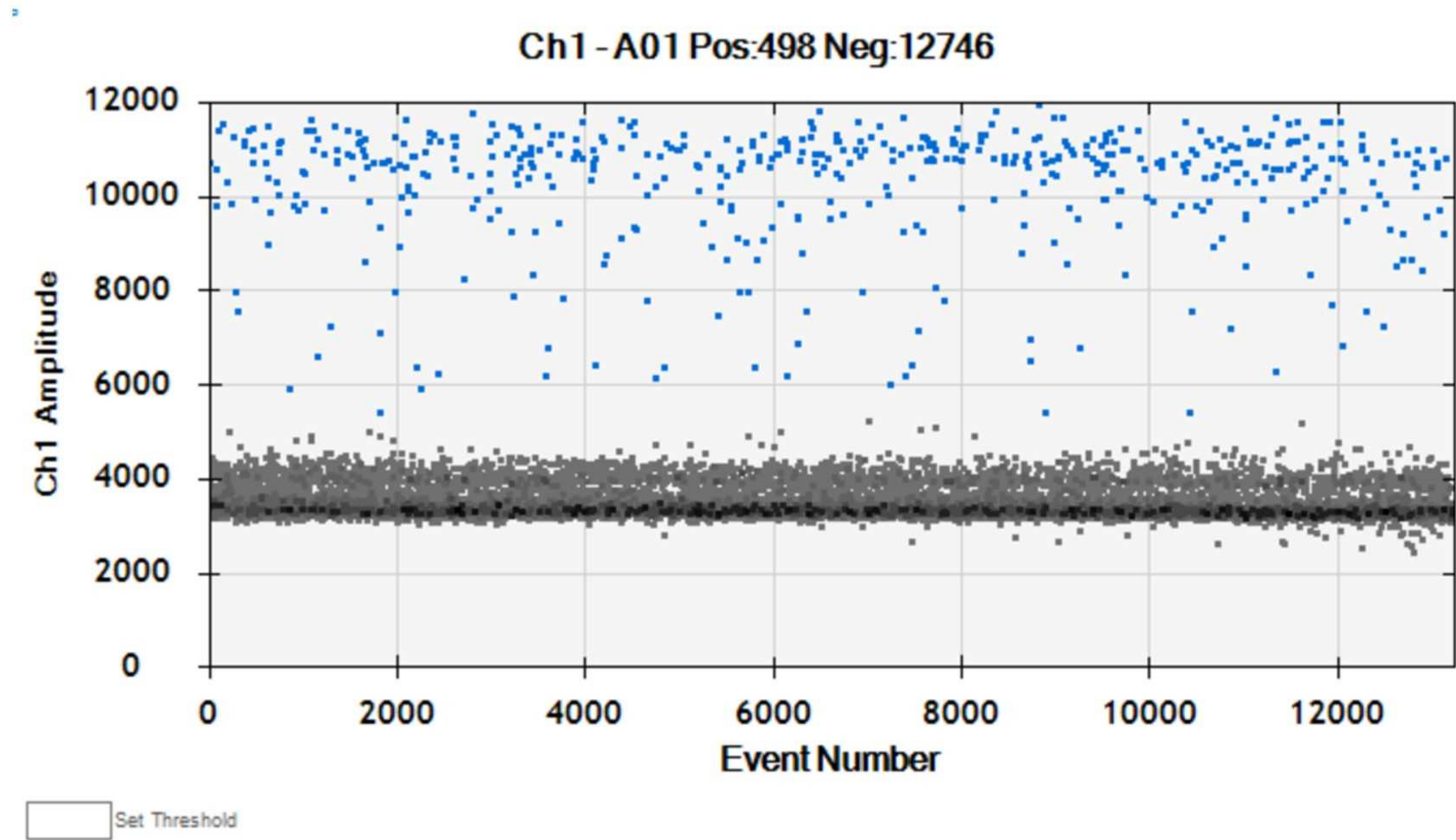
Mutation	①	②	③	④	⑤
E542K	-	-	-	-	-
E545K	-	-	-	-	-
H1047R	+	-	-	-	-

Supplemental Figure 5

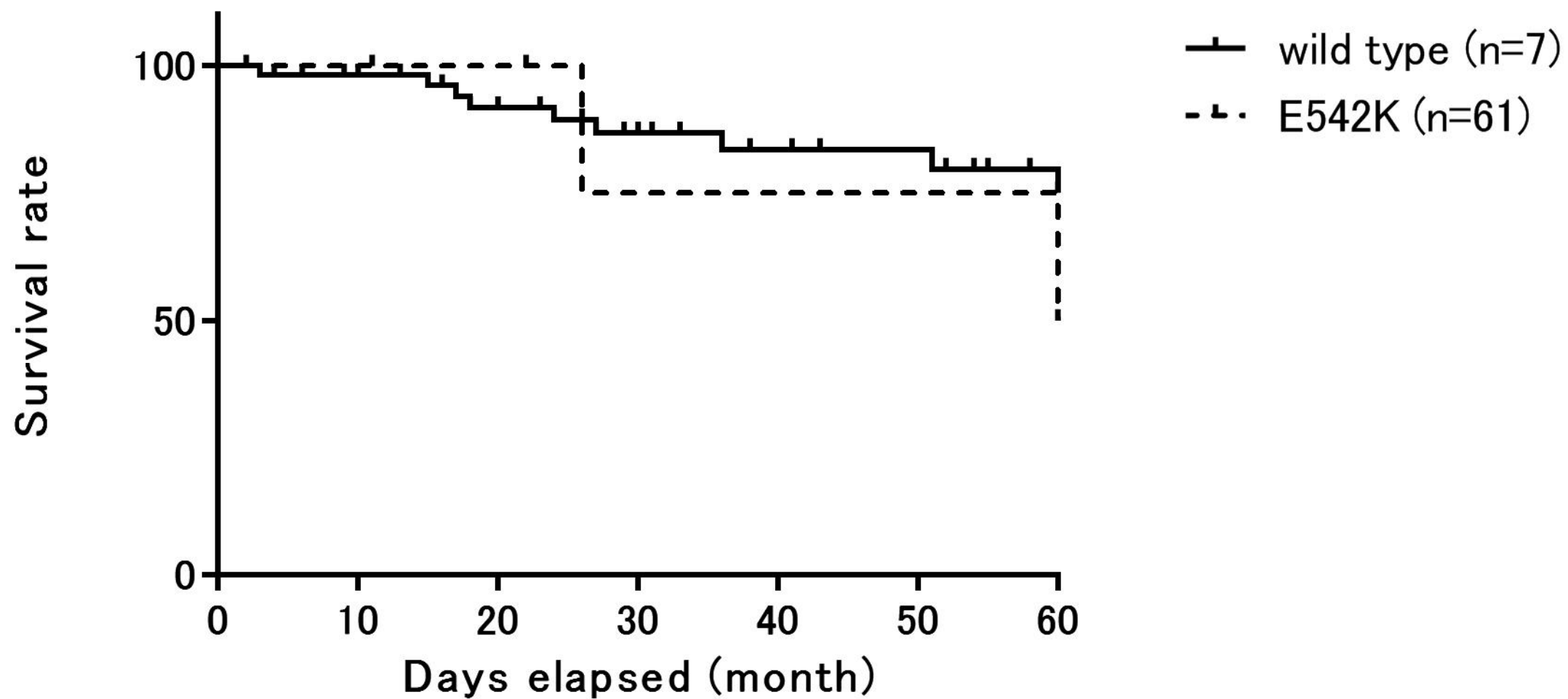
macrodissected tissues



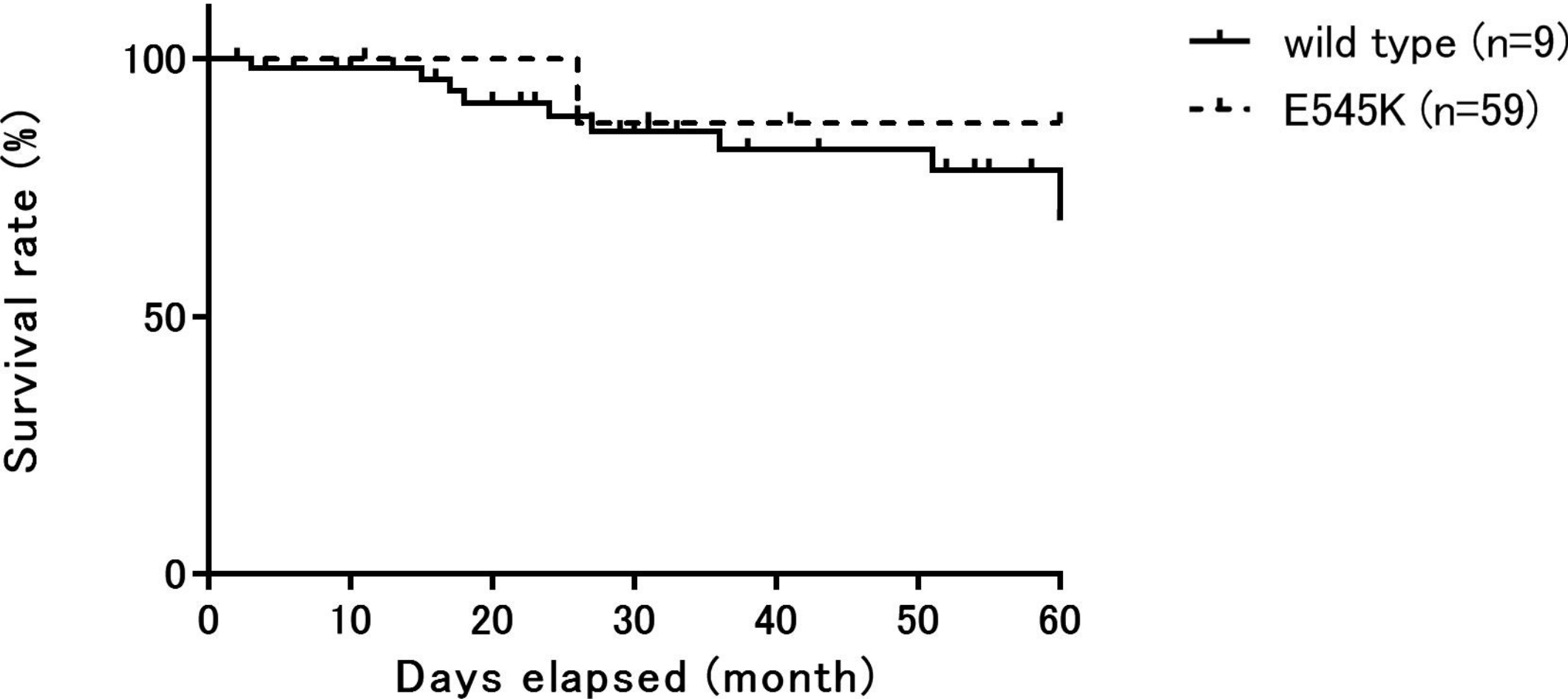
laser-microdissected tissues



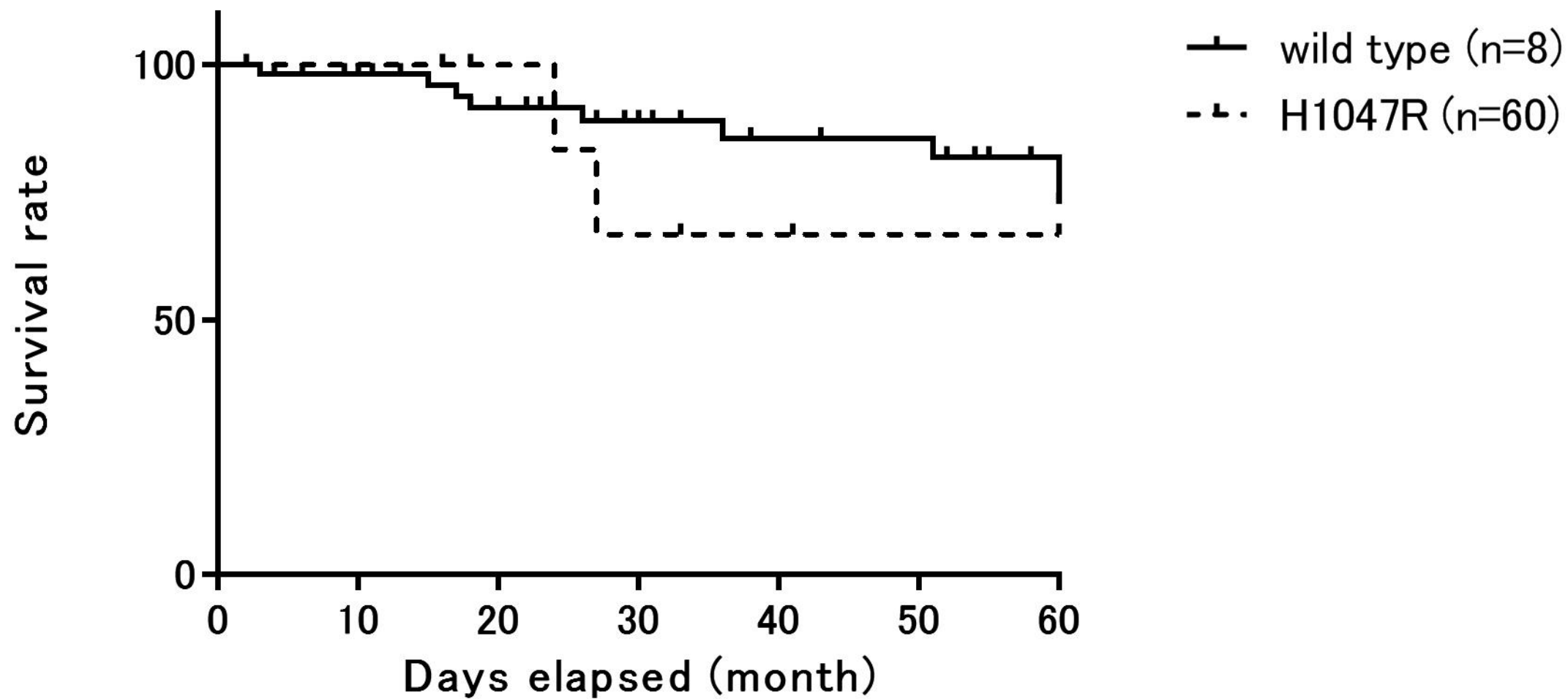
Supplemental Figure 6



Supplemental Figure 6



Supplemental Figure 6



Supplemental Figure 6

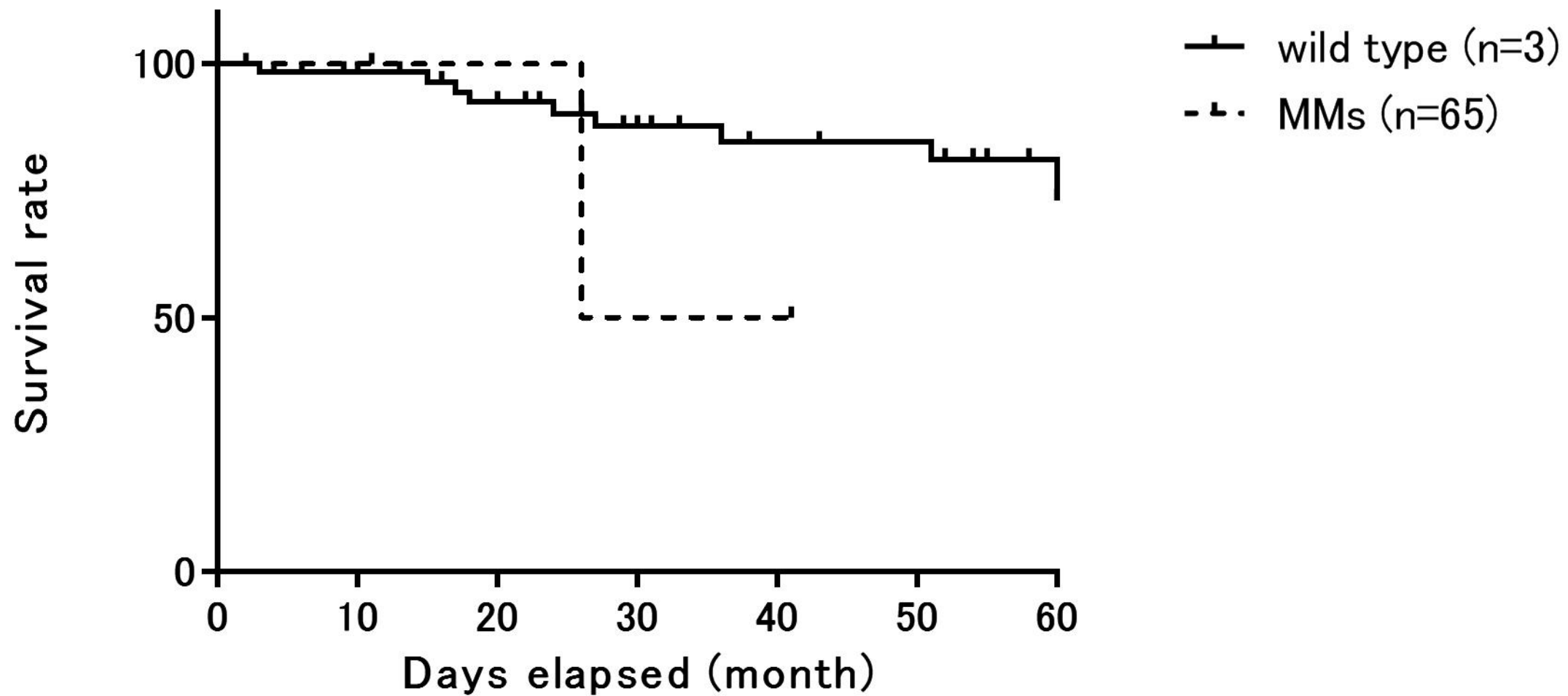


Table1: PIK3CA mutations and clinical manifestations in 68 patients with Extramammary Paget's disease (EMPD).

Case	Age	Sex	<u>Degree of invasion</u>	Lymph node metastasis	Distant metastasis	E542K	E545K	H1047R
1	79	F	invasive	+	-	-	-	-
2	68	F	in situ	-	-	+	-	-
3	82	F	invasive	-	-	+	-	-
4	74	M	invasive	+	+	-	-	-
5	67	F	in situ	-	-	-	-	-
6	57	M	in situ	-	-	-	-	-
7	81	M	in situ	-	-	-	-	-
8	83	M	in situ	-	-	-	-	+
9	72	M	invasive	+	+	+	+	-
10	84	M	in situ	-	-	-	-	-
11	81	F	invasive	+	-	-	-	-
12	55	F	invasive	+	+	-	-	-
13	68	M	invasive	+	+	-	-	-
14	78	M	invasive	+	+	-	-	+
15	92	F	in situ	-	-	-	-	-
16	85	F	invasive	+	-	-	-	-
17	67	M	in situ	-	-	-	+	-
18	69	M	in situ	-	-	-	+	-
19	80	M	in situ	-	-	-	-	+
20	79	F	in situ	-	-	-	-	-
21	81	M	in situ	-	-	+	+	-
22	82	M	in situ	-	-	-	-	-
23	83	M	invasive	-	-	-	-	-
24	87	F	in situ	-	-	-	-	-
25	63	F	in situ	-	-	-	-	-
26	85	M	invasive	+	-	-	-	-
27	52	M	in situ	-	-	-	-	-
28	90	M	in situ	-	-	-	-	-
29	69	M	in situ	-	-	-	-	-
30	76	F	in situ	-	-	-	-	-

31	71	F	in situ	-	-	-	-	-
32	85	M	in situ	-	-	+	-	-
33	76	M	in situ	-	-	-	-	-
34	71	F	in situ	-	-	-	-	-
35	78	M	in situ	-	-	-	-	-
36	78	M	in situ	-	-	-	-	-
37	73	F	in situ	-	-	+	-	-
38	64	F	in situ	-	-	-	-	-
39	65	F	in situ	-	-	-	-	-
40	60	M	in situ	-	-	-	-	-
41	76	F	in situ	-	-	-	-	+
42	77	F	in situ	+	-	-	-	-
43	88	F	in situ	-	-	-	-	+
44	86	F	in situ	-	-	-	-	-
45	84	F	in situ	+	-	-	+	+
46	59	M	in situ	+	-	-	-	-
47	78	F	in situ	-	-	-	-	-
48	93	F	in situ	+	-	-	-	-
49	84	F	in situ	-	-	-	-	+
50	74	F	in situ	-	-	+	-	-
51	60	F	in situ	-	-	-	-	-
52	90	M	in situ	-	-	-	-	-
53	71	F	in situ	-	-	-	+	-
54	59	M	in situ	-	-	-	-	-
55	63	M	in situ	-	-	-	+	-
56	83	F	in situ	-	-	-	+	-
57	64	F	in situ	-	-	-	+	-
58	91	M	in situ	-	-	-	-	-
59	70	M	in situ	-	-	-	-	-
60	66	M	invasive	-	-	-	-	-
61	81	M	in situ	-	-	-	-	-
62	90	F	in situ	-	-	-	-	-
63	78	F	in situ	-	-	-	-	-
64	66	M	in situ	-	-	-	-	-
65	64	F	in situ	-	-	-	-	+

66	71	F	in situ	-	-	-	-	-
67	77	M	in situ	-	-	-	-	-
68	66	M	in situ	-	-	-	-	-

Table 2. Correlations between PIK3CA mutations and clinical findings

Clinical findings	E542K			E545K			H1047R			MMs		
	-	+	<i>P</i> -value	-	+	<i>P</i> -value	-	+	<i>P</i> -value	-	+	<i>P</i> -value
Age (years : mean ± SD)	76.4±5.8	74.8±10.2	0.352	72.7±7.6	75.3±10.2	0.212	79.6±6.9	74.4±10.1	0.079	79.0±5.1	74.8±10.0	0.231
Sex (male : female)	3:4	31:30	0.500	5:4	29:30	0.500	3:5	31:29	0.709	2:1	32:33	0.500
Degree of invasion (in situ : invasive)	5:2	51:10	0.359	8:1	48:11	0.500	7:1	49:11	>0.999	2:1	54:11	0.447
Lymph node metastasis (- : +)	6:1	49:12	0.598	7:2	48:11	0.550	6:2	49:11	0.478	1:2	54:11	0.091
Distant organ metastasis (- : +)	6:1	57:4	0.429	8:1	55:4	0.520	7:1	56:4	0.476	2:1	61:4	0.208
5-year survival rate (% - : +)	84.7	71.4	0.780	82.5	88.9	0.481	75.0	84.5	0.541	84.1	66.7	0.225