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Clinical and molecular analysis of synchronous double lung cancers

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30 **Summary**

Background: Since multiple lung cancer treatment strategies differ, it is essential for clinicians to be able to distinguish between separate primary lesions and metastasis. In the present study, we used array comparative genomic hybridization (aCGH) and somatic mutation (epidermal growth factor receptor: EGFR) to analyze genomic alteration profiles

35 in lung cancer patients. To validate the consistency among the pathological assessments and clarify the clinical differences between double primary lesions and metastasis, we also examined synchronous double lung cancer clinical data. *Methods:* Between January 1970 and March 2010, 2215 patients with lung cancer underwent surgical resection at Nagasaki University Hospital. We performed molecular analysis of 12 synchronous double lung

40 cancer patients without lymph node metastasis (intrapulmonary metastasis in the same lobe (pm1): n=6, primary: n=6). We then evaluated the clinical outcomes of patients with pathologically diagnosed synchronous double lung cancers (intrapulmonary metastasis (pm): n=80, primary: n=39) and other T3 tumors (n=230). *Results:* Examination of the concordance rate (CR) of the copy number changes (CNCs) for paired tumors showed that

45 the metastasis group was larger than the primary group (55.5% vs. 19.6%, $p=0.04$).

Pathological diagnosis and molecular classification were the same in 10 out of 12 cases

(83%). As compared to the primary group, there tended to be an inferior 5-year survival

curve for the pm group. However, in N0 patients, the survival curve for the pm group

overlapped the primary group, while the survival rate of the pm1 group was much higher

50 than that of other T3 group ($p<0.01$). *Conclusions:* Both pathological and molecular

assessment using aCGH adapted in the current study appeared to have a consistency.

Pathological pm1(T3)N0 patients may have a better prognosis than other T3N0 patients.

Key words:

synchronous double cancers

55 intrapulmonary metastasis

non small cell lung cancer

array comparative genomic hybridization

epidermal growth factor receptor

diagnosis

60 prognosis

Introduction

With the development of higher-resolution chest imaging techniques over the past
65 decade, there has been a rising number of multiple lung cancer patients that are being
diagnosed [1]. The crucial issue regarding multiple lung cancers is whether they represent
separate primary lesions or metastasis, as this affects both the stage assessment and the
planning of subsequent treatments. If paired tumors are diagnosed as primary lesions, the
main treatment will be surgery, as these are considered to be a local disease. However,
70 when metastasis is found in these patients, they are automatically classified as T3, for
which surgery and additional chemoradiotherapy will be performed due to this being a
systemic disease.

The clinical and pathological criteria used to define multiple primary lung tumors were
initially published by Martini and Melamed in 1975 [2]. These criteria are based on tumor
75 locations and histological findings. However, clinicians empirically know that some cases
do not completely meet the criteria. For example, metastasis is defined as having multiple
tumors within the same segment that are histologically similar. However multiple

squamous cell carcinomas in the fibrotic lung sometimes arise within the same area. In addition, bronchioloalveolar carcinomas often show multiple ground-glass attenuation
80 within the same segments and thus, these are commonly defined as multiple primary lung cancers.

Since the 1970s, a variety of reliable and powerful molecular tools, such as immunohistochemical and molecular analysis, have become available for evaluating clonal relationships between multiple tumors [3-6]. Mutational analysis of epidermal
85 growth factor receptor (EGFR) and Kirsten rat sarcoma 2 viral oncogene homolog (KRAS) mutation [7] have proven to be powerful tools that can be used in differentiating double primary lung adenocarcinoma from metastatic lesions. Genomic DNA copy number alterations have also been shown to be key genetic events in the development and progression of human cancers and therefore, array comparative genomic hybridization
90 (aCGH) can be utilized during diagnostic procedures [8,9]. When the fluorescence ratio of the aCGH test along with the reference hybridization signals are compared to the reference diploid genome, this yields information on the relative copy number of the sequences in

the test genome. A series of aCGH studies have examined various organs, with special focus on breast tumors [10-15]. Results have demonstrated that this is a powerful method
95 for undertaking comprehensive genome-wide searches, in addition to being an attractive strategy that can be used to define true recurrences among multiple tumors [5, 16]. Nonetheless, there have been few studies specifically designed to examine use of this aCGH technology in lung cancers [17].

In the new 7th TNM staging system guideline (Union of International Cancer Control:
100 UICC. Ver. 7) [18], pm1 was downgraded from T4 to T3, while metastasis in other lobes on the same side (pm2) was downgraded from M1 to T4. Therefore, this new TNM staging system could potentially lead to changes in multiple lung cancer patient prognosis.

To validate clinicopathological assessment and clarify the difference between double primary lesions and pulmonary metastasis, we analyzed genomic profiles of 12 cases that
105 were clinicopathologically defined as double primary lesions (n=6) or metastasis (n=6). To exclude the impact of the major prognostic factor of lymph node metastasis, we focused on pathological N0 patients and analyzed clinical data from postoperative synchronous

double lung cancer patients.

110 **Materials and Methods**

Patients

Between January 1970 and March 2010, a series of 2215 patients with lung cancer underwent surgical resection at Nagasaki University Hospital. A total of 119 patients (5.8%) had synchronous double lesions, with 80 diagnosed as intrapulmonary metastatic NSCLC (pm) and 39 as double primary NSCLC (primary). Out of 119 patients, 66 were classified as N0, with 28 having double primary and 38 having pm. As per the 7th TNM classification guideline, 145 patients met the T3N0 criteria, including 32 who were classified as pm1N0. **Table 1** shows the clinical characteristics for the 12 patients (24 tumor samples) examined for the genomic and mutation profiling.

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Pathological Evaluation

Samples classified as “primary” had to 1) have a different histological subtype, 2) be different or exhibit clear differentiation in paired tumors, 3) have undergone different

immunostaining experiments, for example thyroid transcription factor 1 (TTF-1),
125 cytokeratins 7 (CK7), and 20 (CK20), or 4) have low grade lymphatic and blood vessel
invasion. All of the pathological materials available for tumor classification were reviewed
by three expert pathologists using the standard World Health Organization Classifications
[19]. Disease status was staged according to the TNM staging system (UICC Ver. 7).
Tumor locations were used as supportive information.

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Tissue Procurement

All patients provided informed consent for the use of tumor specimens, and the Ethics
Review Board on Clinical Research of Nagasaki University Hospital approved the study
protocol (#10072868). Tumor specimens were obtained from patients with lung cancer
135 who underwent surgical resection at Nagasaki University Hospital from January 1970 to
March 2010. Among the patients having their synchronous double lung cancer resected
operatively, cases in which the tumor specimen contained more than 70% cancer cells
were saved for further molecular examinations. A total of 12 cases with 24 tumors were
included in the study. At the time of the surgeries, study patients were considered to have

140 clinicopathological findings of either double “primaries” or “pm”. DNA was extracted
from formalin-fixed, paraffin-embedded block tissue using the QIAamp DNA Mini Kit
(Qiagen, Germany) in accordance with the previously reported manufacturer’s protocol
[20]. The detailed method is described in **Supplemental Text I**.

145 *Genomic Profiling*

Genomic DNA extracted from samples and the reference female genomic DNA
(Promega, Madison, WI, USA) were chemically labeled with Cy5 and Cy3, respectively,
using the Genomic DNA ULS Labeling Kit (Agilent Technologies, USA) and hybridized
to Agilent 60K comparative genomic hybridization arrays according to the manufacturer’s
150 instructions. Images of microarrays were scanned by an Agilent DNA Microarray Scanner,
and raw microarray image files were generated using the Feature Extraction Software
(version 9.5). Agilent Genomic Workbench (version 5.0) was used to visualize, detect and
analyze chromosomal changes, with the ADM-2 algorithm threshold set to 5.0. We defined
a log₂ ratio more than 0.25 as a gain, and less than -0.25 as a loss. The total length of the

155 chromosomal aberrant region, which is the sum of each segment gained or lost, was used
as an indicator of genomic instability.

The patterns of gains and losses for each pair of tumors were compared. Since the
concordance of the copy number changes (CNCs) between each of the paired tumors have
been used as diagnostic values in several previous studies [8,10,21], we used these to
160 clarify whether cases were primary or metastasis. The concordance rate (CR) was defined
as the ratio of the length of the chromosomal aberrant region between two tumors, which
was essentially a modified value of measure M that was derived from a percent
concordance calculation by Waldman et al. [10,22]. This value was the percentage of the
length of overlapping CNCs divided by the average length of the CNCs for each pair of
165 tumors. For an (i,j) pair, this would be calculated as follows:

$$CR(i,j) = \frac{\#(L_i \cap L_j)}{1/2 \times (\#L_i + \#L_j)} \times 100,$$

in which L_i and L_j are the length of the chromosomal aberrant region of the (i,j) pair.

The mean CR for all pairs of tumors that came from different patients in this study,
and which therefore were definitively independent, was 11.4% (range, 0.001% to

170 75.76%). **Supplemental Figure I** shows the histogram that was created using the data. We defined 50% as a criterion of diagnosis since 52% was the border range within the upper 95% of this histogram. Tumor pairs were classified as metastasis when the rate exceeded the criterion.

175 *Mutational Profiling*

The hot-spot mutations of *EGFR*, along with the 9- to 18-bp deletions in exon 19 and the L858R missense mutation in exon 21 were analyzed using the mutant-enriched polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method [23]. Primer pairs used for PCR in this study are shown in **Table 2**, with the detailed method described in **Supplemental Text II**.

Statistical Analysis

Overall survival (OS) and Disease free survival (DFS) was assessed using the Kaplan-Meier method. The influence of classifications was studied using the log-rank test. Comparisons of results were examined using the Mann-Whitney *U* test, Kruskal-Wallis

test, Welch's t test, and χ^2 test. Results were considered significant at the 0.05 level. SPSS version 17 software (SPSS Japan, Tokyo, Japan) was used for the analyses.

Results

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Molecular Analysis Performed by Array CGH and Mutational Profiling of EGFR

To evaluate the pathological diagnoses as double primary or metastasis, we conducted aCGH analyses on the 24 tumor pairs from the 12 cases in the N0 group. Both double primary (n=6) and metastatic (n=6) cancers were observed. The aCGH genomic profiles for each of 12 cases are shown in **Supplemental Figure II. Figure 1** shows a typical aCGH result. Amplification regions were noted in both tumors in case 2, while case 7 displayed amplification in one of the tumors on 5p. The paired tumors seen in case 2 exhibited similar genomic alterations, which suggest a diagnosis of metastasis. However, the different alterations seen in case 7 suggest a diagnosis of double primary.

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200 We calculated the total number and size of the CNCs along with the CR between each pair of tumors. Cases pathologically diagnosed as metastasis exhibited a statistically higher CR as compared to that seen in the double primary groups (55.5% vs. 19.6%, $p=0.04$). Using the results of aCGH, we cytogenetically diagnosed the 12 cases as either double primary or metastases. A total of 6 patients were diagnosed as double primary
205 while the other 6 were metastasis (**Table 3**).

We also performed hot-spot mutation analysis of *EGFR* and detected mutations of either exon 19 or 21 in 8 out of the 12 cases (66.7%). A previous study found that double primary cancer occurred when there were different mutations between the paired tumors, while metastasis occurred in cases with the same mutation [7]. Based on these findings,
210 the mutation analysis in the current study indicated that 4 cases were double primary cancers, 4 were metastasis, and 4 were equivocal (**Table 3**). Results for 7 out of 12 of these cases (58.3%) were identical to those of the pathological examinations.

Comparison of Pathology and Molecular Profiling

215 **Table 3** summarizes the results for the classification schemes. While the aCGH and
mutational analysis results were comparable, the molecular diagnosis had to be determined
using the results of aCGH, as the mutational analysis was ambiguous. Molecular diagnosis
classified 6 cases as double primary and 6 cases as metastasis. The overlap between the
pathological diagnosis and the molecular classification was 83% (10/12 cases), with a
220 statistically strong correlation noted between the two ($p=0.04$). There were 2 contradicted
cases (cases 1 and 11). Because of a similar histology with the well-differentiated
papillary type, the pathological diagnosis for case 1 was pm1. However the CR was only
2.6%. Since detailed pathology for case 1 indicated the primary and secondary lesion
tumor sizes were 20 and 12 mm without lymphatic and blood vessel invasion, we
225 considered this case to be double primary. Case 11 was also double primary, as it had a
lesion with a mucinous component in the papillary background, along with another lesion
that showed a mucinous invasive adenocarcinoma with a small moderately differentiated
component. However, since the CR was high (68.8%) in this case, there is a possibility
that the mucinous component might have metastasized from the original lesion.

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Patient Characteristics

Comparisons of the pathologically diagnosed double primary and intrapulmonary metastatic groups indicated the intrapulmonary metastasis cases had a significantly larger tumor size ($p=0.04$), higher frequencies of lymph node metastasis ($p<0.01$) and pleural invasion ($p=0.02$). In addition, most of the intrapulmonary metastasis was observed in the same lobe ($p<0.01$). In the double primary group, smoking was more frequently observed as compared to the metastasis group ($p=0.04$) (data not shown). When NO patients were compared, only the location proved to be a significantly different clinical factor. Furthermore, double primary lesions were often observed in a different lobe while intrapulmonary metastasis was usually observed in the same lobe ($p<0.01$) (**Table 4**).

Survival Curves for the Double Primary and Metastasis Groups

Metastasis patients tended to have an inferior overall 5-year survival curve as compared to the double primary group ($p=0.26$) (Fig. 2a). However, when focusing on the

245 pm1N0 patients, the patient OS and DFS curves for pm1 and double primary cancers
crossed, with the 5-year OS higher than 68% in both groups ($p=0.14, 0.93$) (Fig. 2b,c). It
was also notable that the 5-year OS rates for pm1N0 patients were extremely higher than
the rates seen for the other T3 patients ($p<0.01$) (Fig. 2d).

250 **Discussion**

To evaluate the accuracy of pathological diagnoses, we performed an aCGH analysis
and then determined the cytogenetic and mutational profiles of synchronous double
NSCLC. Notably, our results showed the CR of the CNCs of the paired tumors in the
pathological pm1 group were significantly higher than that seen for the pathological
255 double primary group. This suggests primary tumors are a molecularly different neoplasm.

With regard to the diagnosis, 10 out of 12 (83%) patients had the same molecular and
pathological diagnosis, which is comparable to a previously published study [24].

Although there were 2 cases with conflicting diagnoses (cases 1 and 11), the detailed
pathological analyses supported the aCGH results. Pathological diagnoses are based on

260 subjective assessments. In contrast, the cytogenetic profiles were calculated using an aCGH analysis, which objectively attempts to determine whether the tumors are monoclonal or not [9-16]. Therefore, we assume the aCGH analysis should exhibit less variance and provide superior pathological results in these limited difficult cases.

Girard et al., however, has reported there are several drawbacks to using aCGH in
265 normal clinical practices. First, the procedure requires fresh frozen tissue and large amounts of genomic DNA. In the present study, we demonstrated that formalin-fixed, paraffin-embedded tissues can be used in aCGH studies, which improves the tissue handling aspect. Second, aCGH is still expensive and can be both a time-consuming and labor-intensive procedure. Therefore, although the aCGH analytical results appear to be
270 preferable to the pathological diagnostic results, the pathological diagnostic workup remains the first-line tool for differentiating between double primary and pm in the clinical practice.

With regard to mutational profiling, there may be several limitations. In a case with two tumors having matched EGFR mutations, we assume the tumors are metastasis

275 because theoretically metastatic lesions inherit genomic characteristics in theory. However, it has been previously suggested that matched mutations may occur in double primary while additional mutations may occur in metastasis [25]. Additionally, several studies have reported finding intratumor heterogeneity of the EGFR mutation [17, 26-28]. Moreover, since 4 out of the 12 paired tumors harbored no mutations in either tumor, it
280 was impossible to determine the clonal relationships. Because of this, we were only able to diagnose 1 out of 12 cases as being irrefutable double primary in the present study (case 12, **Table 2**). These previous findings are in line with the ambiguity of the mutational analysis. Thus, as has been previously suggested, utilization of a specific molecular alteration, which includes KRAS, P53, and EML4-ALK, is required to make a more
285 precise diagnosis [21].

We have shown that the 5-year survival rates for synchronous double primary and intrapulmonary metastasis patients are similar in the N0 patients, although the theoretical prognosis of pm1 patients appears to be poorer than that for the double primary patients because pm1 is classified as T3 and most of the double primary in the present study was

290 classified as T1 (**Table 1**). Additionally, the pm1 5-year survival rates in N0 patients were much better than the rates found for other T3 patients. Because of the unexpectedly favorable prognosis found for the pm1n0 patients, we decided to reanalyze the survival data based on the definition of Martini and Melamed. In addition, we also reexamined the data via the comprehensive histologic subtyping assessment methodology, which has been

295 reported by Travis et al. to be a very accurate histological analysis for multiple lung adenocarcinoma diagnosis [29]. While our reanalysis found that the diagnosis changed in 5 out of 119 cases, the results of the overall survival and disease free survival curves remained similar (data not shown). Therefore, we can conclude that our survival data was reliable. Theoretically, intrapulmonary metastasis can occur at the micro lymphatic or

300 blood vessel implantation before hilar lymph node metastasis. In such cases, single lobectomy might be sufficient for the purpose of complete resection. Detailed analyses of the clinicopathological data of the 32 pm1N0 patients showed that with the exception of one patient, all of the metastatic tumor diameters were less than 3 cm. Therefore, we postulate that most of the small-sized pm1N0, especially those less than 3 cm, could have

305 remained to some extent within the lung, thereby leading to an undue influence on the diagnosis. Specific information on the T grade of the small-sized pm1N0 might ultimately be the information needed to help clinicians better decide on a suitable therapeutic plan that includes both surgery and chemotherapy.

310 **Conclusion**

We showed the usefulness of the aCGH assessment in helping to differentiate between double primary lesions and metastatic lesions, in addition to improving the accuracy of the clinicopathological diagnosis. However, since the prognosis of the double primary group and metastasis group proved to be similar, this suggests that the small pm1 does not play an important role in determining the prognosis of patients with NSCLC. Furthermore, the better prognosis of pm1 group as compared to that in other T3 group in N0 patients indicates that use of small pm1 as a T3 factor can result in an overestimation of the prognosis. Detailed investigations that specifically examine the size

and number of lesions that occur during intrapulmonary metastasis will need to be
320 undertaken in order to definitively confirm the present findings.

Conflict of Interest Statement:

None declared.

No financial or other potential conflicts of interest exist for any of the authors.

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Table 1: Clinicopathological factors of the analyzed patients

No. / age / sex	status		main tumor / another tumor				dominant diagnostic factor	DFS	
	MM	pathology	location	size (mm)	histology	differentiation		days	outcome
1 : 62 F	pm1	pm1	LUL (S1+2)	20	Adeno	well	subtype	3394	alive
			LUL (S1+2)	12	Adeno	well			
2 : 69 F	pm1	pm1	LUL (S1+2)	15	Adeno	well	vascular invasion	1844	recurrence
			LUL (S1+2)	10	Adeno	well			
3 : 76 M	pm1	pm1	RML (S4)	60	Adeno	well	vascular invasion	218	recurrence
			RML (S4)	9	Adeno	well			
4 : 79 F	pm1	pm1	LUL (S1+2)	42	Adeno	well	subtype	2195	alive
			LUL (S1+2)	6	Adeno	well			
5 : 77 M	primary	pm1	RUL (S2)	45	Adeno	well	subtype	281	recurrence
			RUL (S3)	8	Adeno	well			
6 : 56 M	primary	pm1	RUL (S2)	17	Adeno	moderately	subtype	2920	alive
			RUL (S3)	19	Adeno	moderately			
7 : 65 M	primary	primary	RML (S4)	15	Adeno	moderately	vascular invasion	267	recurrence
			RUL (S2)	16	Adeno	moderately			
8 : 71 M	primary	primary	LLL (S6)	120	Sq	poorly	subtype	1254	alive
			LUL (S1+2)	25	Sq	moderately			
9 : 72 F	primary	primary	RLL (S6)	18	Adeno	poorly	differentiation	1825	alive
			LLL (S8)	12	Adeno	moderately			
10 : 41 F	primary	primary	RUL (S2)	32	Adeno	well	subtype	1357	recurrence
			LLL (S6)	24	Adeno	moderately			
11 : 57 F	primary	primary	LLL (S6)	32	Adeno	well	subtype	4126	alive
			RLL (S3)	28	Adeno	well			
12 : 61 M	primary	primary	RUL (S3)	33	Adeno	moderately	subtype	297	recurrence
			LUL (S1+2)	25	Adeno	moderately			

Abbreviations: MM: Martini and Melamed Criteria, DFS: disease free survival, LUL: left upper lobe,

LLL: left lower lobe, RUL: right upper lobe, RML: right middle lobe, RLL; right lower lobe,

Adeno: adenocarcinoma, Sq: squamous cell carcinoma.

Table 2: Primers used for polymerase chain reaction

Primer (Primer length)	Sequence
ex19 S-1 (28)	ATCCCAGAAGGTGAGAAAGATAAAAATTC
ex19 AS-1 (21)	CCTGAGGTTTCAGAGCCATGGA
ex19 HR-F (19)	AAAATTCCCGTCGCTATCA
ex19 HR-R (18)	AAGCAGAAACTCACATCG
ex21 f-s (21)	CGCAGCATGTCAAGATCACAG
ex21 r-s (21)	ACTTTGCCTCCTTCTGCATGG
ex21 HR-F (18)	AGATCACAGATTTTGGGC

Table 3: Clinicopathological assessment and molecular profile diagnosis results

Case	status		aCGH		EGFR mutation			DFS	
	pathology	MM	concordance rate (%)	diagnosis	exon19	exon21	diagnosis	days	outcome
1 A/B	pm1	pm1	2.6	primary	(+)/(+)	(-)/(-)	metastasis	3394	alive
2 A/B	pm1	pm1	67.3	metastasis	(+)/(+)	(-)/(-)	metastasis	1844	recurrence
3 A/B	pm1	pm1	61.6	metastasis	(-)/(-)	(-)/(-)	equivocal	218	recurrence
4 A/B	pm1	pm1	63	metastasis	(-)/(-)	(+)/(+)	metastasis	2195	alive
5 A/B	pm1	primary	87	metastasis	(-)/(-)	(+)/(+)	metastasis	281	recurrence
6 A/B	pm1	primary	51.2	metastasis	(-)/(-)	(-)/(+)	primary	2920	alive
7 A/B	primary	primary	11	primary	(-)/(-)	(-)/(+)	primary	267	recurrence
8 A/B	primary	primary	0.3	primary	(-)/(-)	(-)/(-)	equivocal	1254	alive
9 A/B	primary	primary	10.9	primary	(-)/(-)	(-)/(+)	primary	1825	alive
10A/B	primary	primary	23.2	primary	(-)/(-)	(-)/(-)	equivocal	1357	recurrence
11 A/B	primary	primary	68.8	metastasis	(-)/(-)	(-)/(-)	equivocal	4126	alive
12 A/B	primary	primary	3.4	primary	(-)/(+)	(+)(-)	accurate primary	297	recurrence

Abbreviations: A: primary tumor, B: secondary tumor, MM: Martini and Melamed Criteria, DFS: disease free survival.

10 **Table 4: Characteristics of patients with synchronous double NSCLC (N0)**

	double primary	pm	p value	especially pm1	p value
	N = 28	N = 38		N = 32	
sex ^a					
male	20	27		23	
female	8	11	0.97	9	0.98
age ^a					
over 70	18	19		17	
under 70	10	19	0.25	15	0.46
smoking ^a					
+	21	22		18	
-	7	16	0.15	14	0.21
location ^b					
same	6	32			
ipsilateral	8	6			
contralateral	14	0	<0.01		
size ^b (mm)					
~30	20	22		18	
31~70	7	11		9	
71~	2	5	0.24	5	0.33
histology ^b					
adeno	20	28		23	
squamous	1	7		6	
other	7	3	0.29	3	0.75
ly factor ^a					
(-)	12	11		11	
(+)	12	25		20	
unknown	4	2	0.13	1	0.36
v factor ^a					
(-)	12	19		18	
(+)	13	16		13	
unknown	3	3	0.63	1	0.52
p factor ^a					
(-)	19	25		23	
(+)	9	12		9	
unknown	0	1	0.98	0	0.79

a : Mann Whitney U test. b : Kruskal Wallis test.

Abbreviations:

pm : intrapulmonary metastasis, pm1: intrapulmonary metastasis to the same lobe.

Figure Legends

Figure 1:

Genomic profiles of 5 chromosome arms. A and B indicate the primary and secondary lesions, respectively. Case 2 profiles (2A and 2B) suggest a diagnosis of metastasis,

5 while case 7 profiles (7A and 7B) suggest double primary lesions.

Figure 2:

a) The overall survival curves for the synchronous double NSCLC patients.

b) The overall survival curves for pm1 and double primary NSCLC patients without lymph node metastasis.

10 c) The disease free survival curves for pm1 and double primary NSCLC patients without lymph node metastasis.

d) The overall survival curves for pm1 and T3 NSCLC patients without lymph node

metastasis.

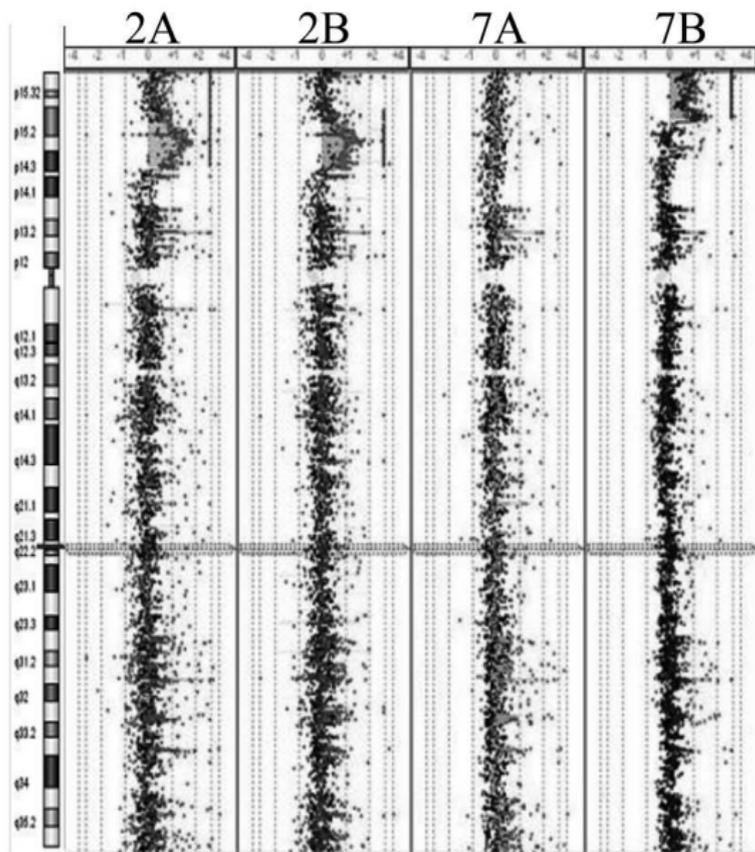


Figure2(a)

Overall survival of NSCLC patients (pm vs. double primary)

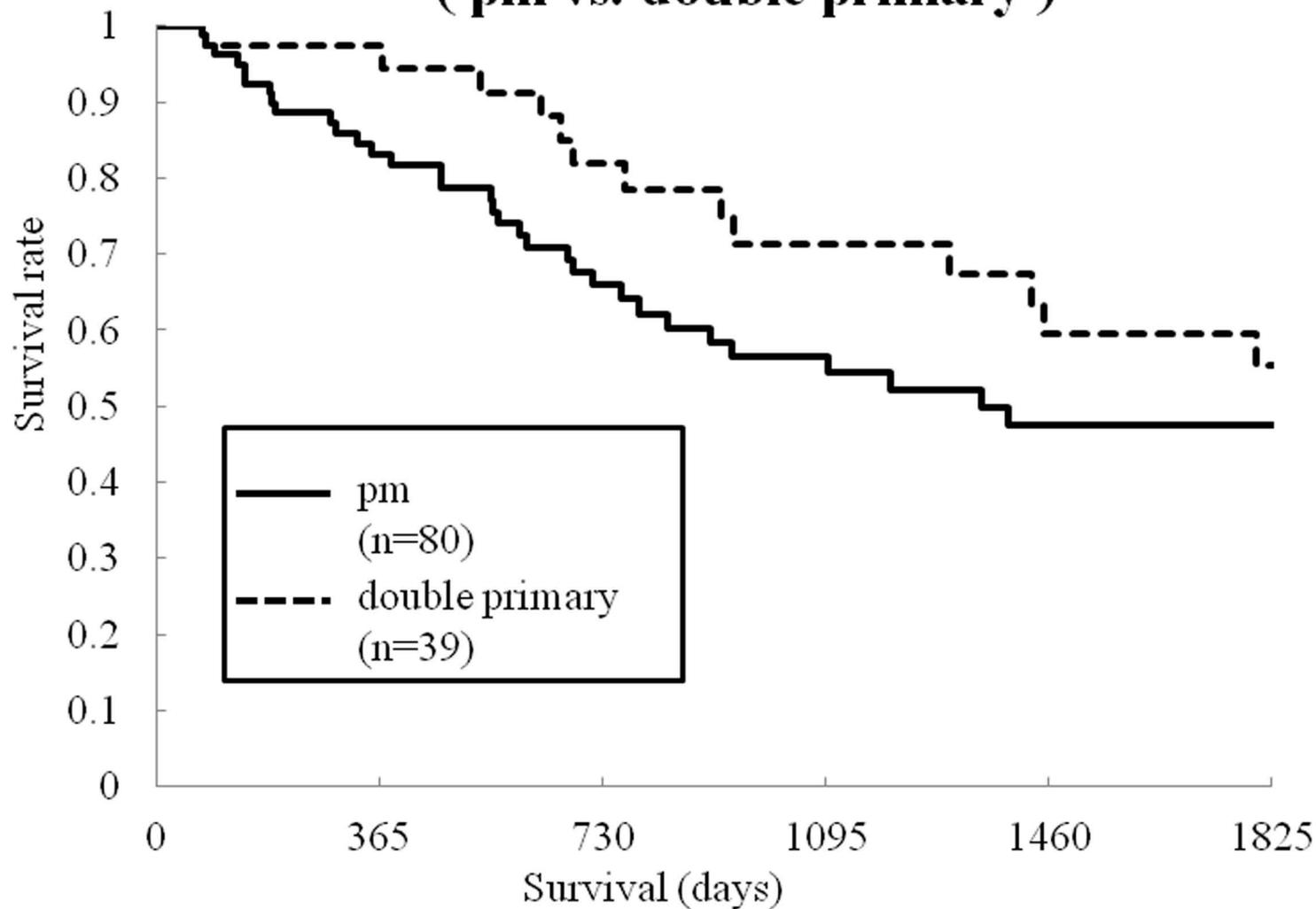


Figure2(b)

Overall survival of NSCLC patients (pm1 N0 vs. double primary N0)

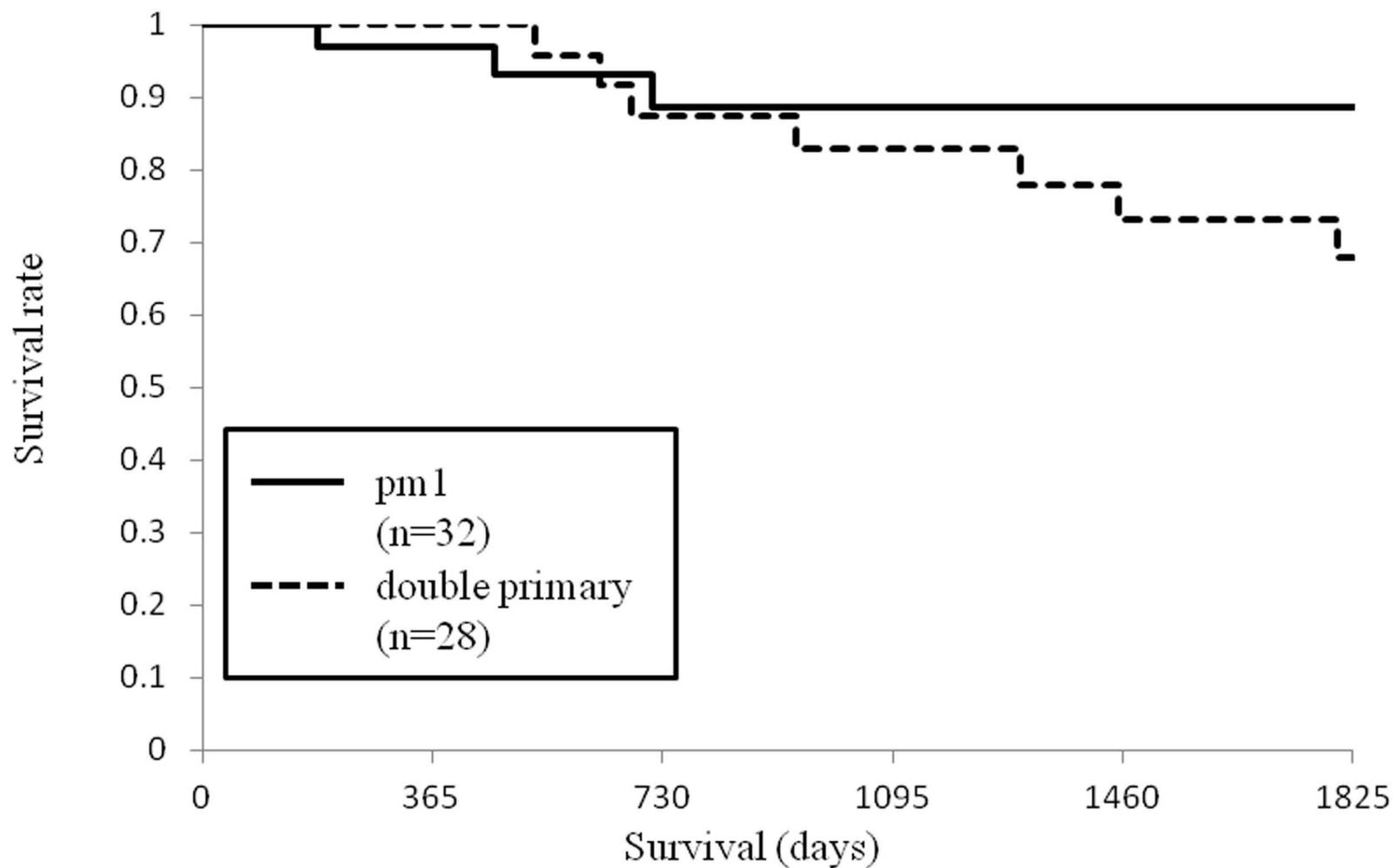


Figure2(c)

Disease free survival of NSCLC patients (pm1 N0 vs. double primary N0)

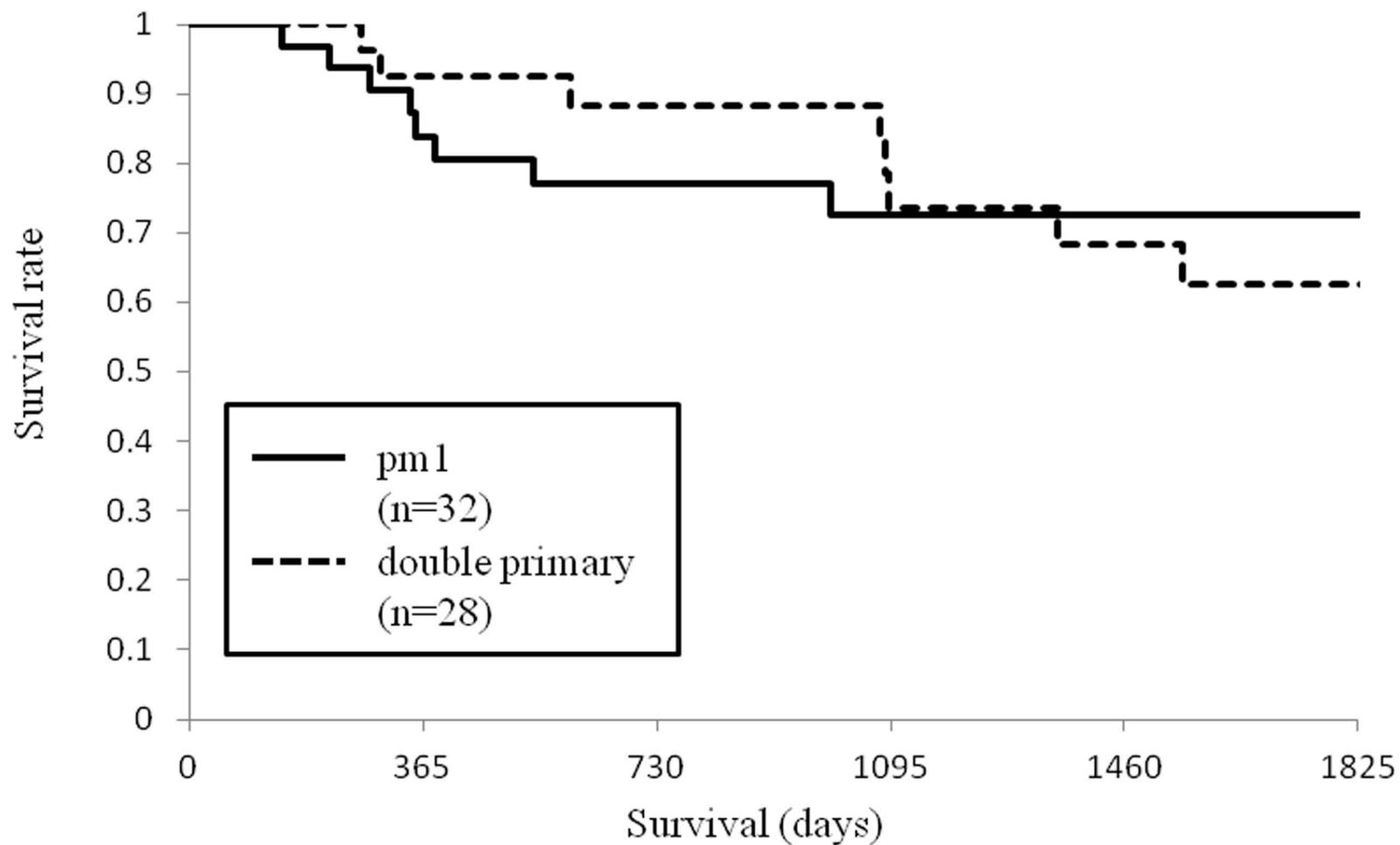
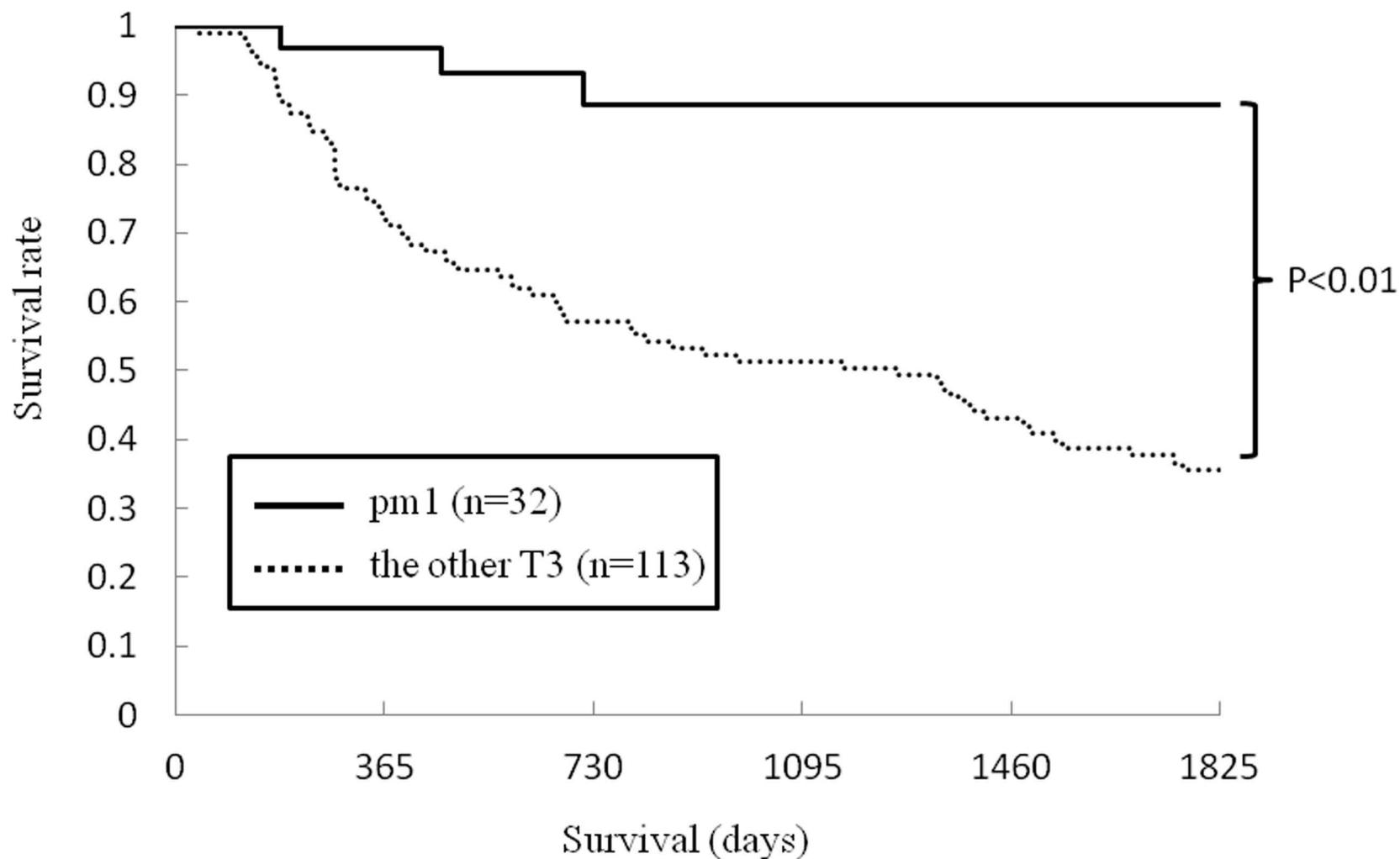


Figure2(d)

Overall survival of NSCLC patients (pm1 N0 vs. the other T3 N0)



Supplemental Text I: Method used for DNA extraction from formalin-fixed, paraffin-embedded block tissue

Five to ten slices of the microdissected tumor area that were 10 μm thick were removed from each formalin-fixed, paraffin-embedded block. These areas were identified using a hematoxylin and eosin stain. After removing the paraffin using 80% xylene, the deparaffinized tissue pieces were washed twice with absolute ethanol, and then spun down. After the pieces were dried, the pellet was resuspended in 360 μL of buffer ATL, incubated at 95°C for 15 minutes, followed by cooling of each sample to room temperature. After adding proteinase K to digest the samples, they were then incubated at 56°C in a rotation oven for three days, with periodic mixing and the addition of proteinase K every 24 hours. After three days, we added 400 μL of buffer AL (equal volume to sample suspension), followed by incubation at 70°C for 10

minutes. Subsequently, we then added 400 μL of absolute ethanol, placed the samples into the spin column, followed by centrifugation at 8000 x g for 1 minute. The column was then washed twice with 500 μL of buffer AW1, centrifuged at 8000 x g for 1 minute, washed once with buffer AW2, and then centrifuged once more time at 14,000 x g for 3 minutes. Genomic DNA was eluted with 55 μL of buffer AE, and 200 μL of buffer TE. Extracted DNA was quantified on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Supplemental Text II: PCR-RFLP method used for the EGFR mutation search

In order to examine the mutation of exon 19, we used ex19 S-1 for the forward primer and ex19 AS-1 for the reverse primer for the 1st PCR. We also used ex21 f-s for the forward primer and ex21 r-s for the reverse primer to examine the mutation of exon 21.

5 DNA was amplified for 35 cycles at 94°C for 30 seconds, at 60°C for 30 seconds, and at 72°C for 30 seconds, followed by 5 minutes of extension at 72°C. For the 1st RFLP, the restriction enzyme MseI was used to digest the TTAA sequence in the wild-type genes, as this is frequently absent in exon 19 deletion mutants and results in the enrichment of deletion-type genes. In addition, the restriction enzyme MscI was used

10 to digest the TGGCCA sequence in the wild-type genes, as the mutant type L858R in exon 21 is not digested due to the base substitution of T to G at the first base of TGGCCA. Because of this, there is enrichment of the L858R mutant. The 1st PCR

product obtained using these restriction enzymes was incubated at 37°C for 12 hours.

For the 2nd PCR, we used ex19 HR-F for the forward primer and ex19 HR-R for the

15 reverse primer in order to examine the mutation of exon 19. To examine the mutation

of exon 21, we used ex21 HR-F for the forward primer and ex21 r-s for the reverse

primer. The 1st RFLP product was amplified for 20 cycles at 94°C for 30 seconds, at

60°C for 30 seconds, and at 72°C for 30 seconds, followed by 5 minutes of extension

at 72°C for the 2nd PCR. For the 2nd RFLP, the restriction enzyme Sau96I was used to

20 digest the GGNCC sequence. Since the wild-type is not digested, even partial digestion

of the PCR product indicates the presence of a mutation in exon 21. The 1st PCR

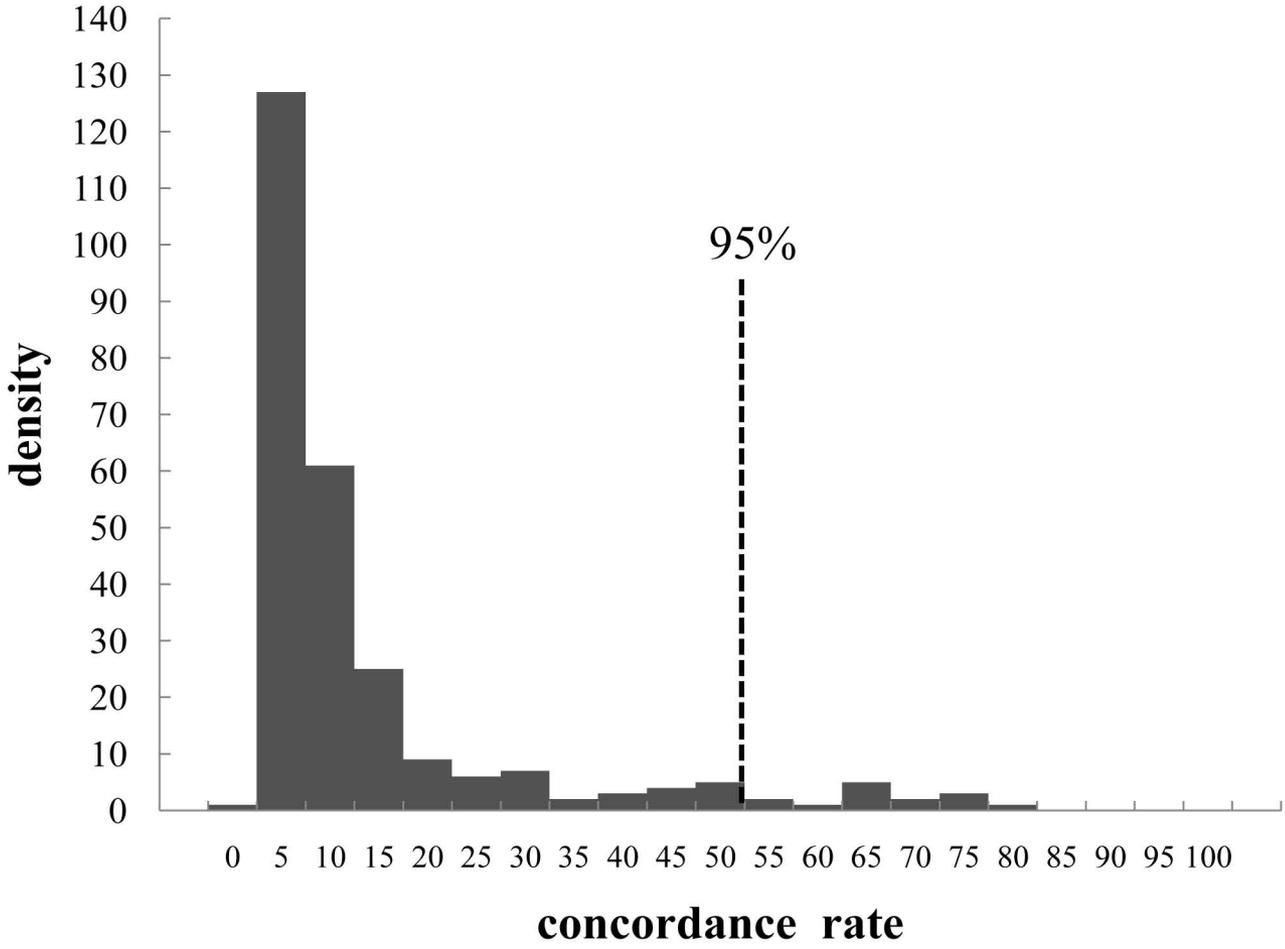
product obtained using these restriction enzymes was incubated at 37°C for 12 hours.

However, this step was not required for the detection of the mutation in exon 19. After

the 2nd PCR or 2nd RFLP, the product was then analyzed on 6% or 8% Polyacrylamide

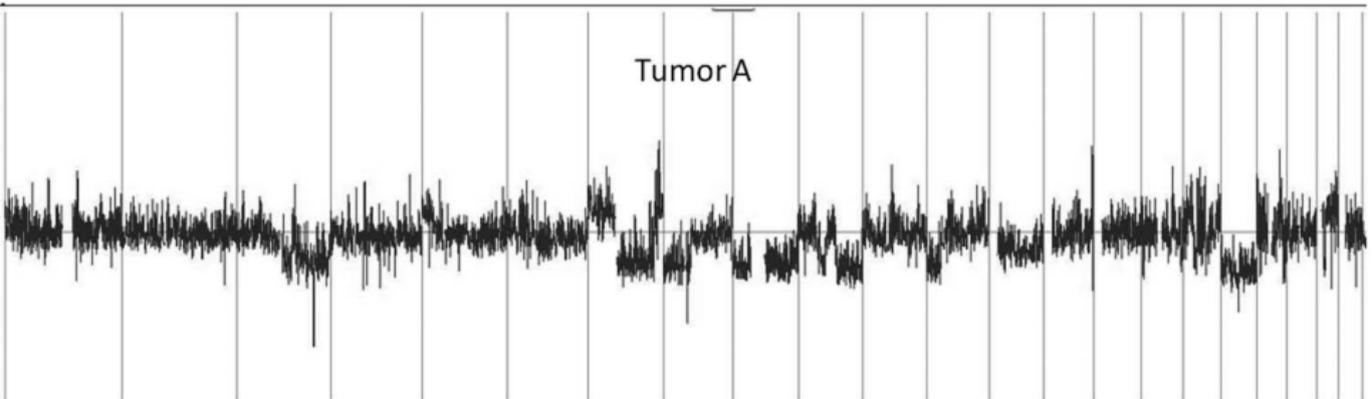
25 Gel Electrophoresis.

Histogram performed on 264 artificial pairs (two different patients) of tumors

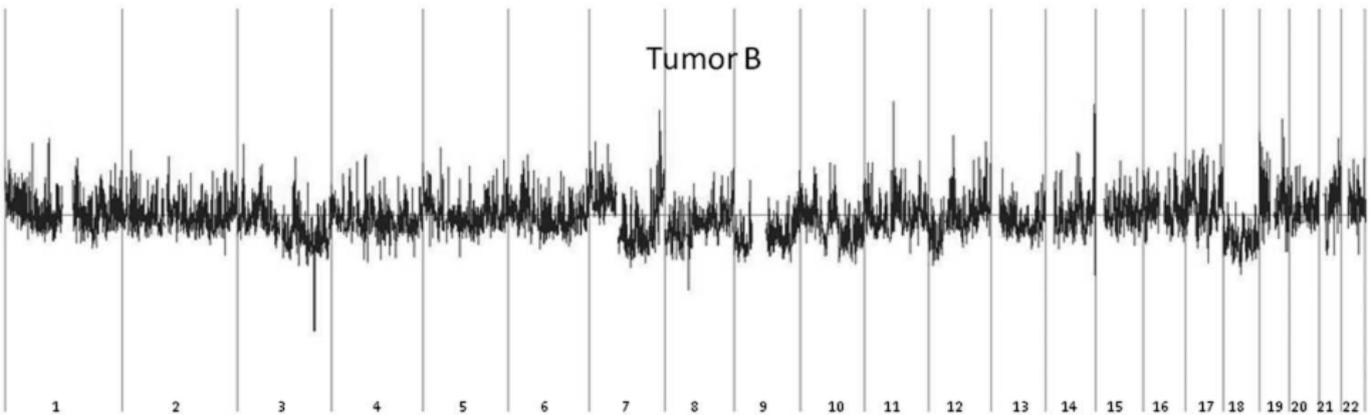


Case 1

Tumor A



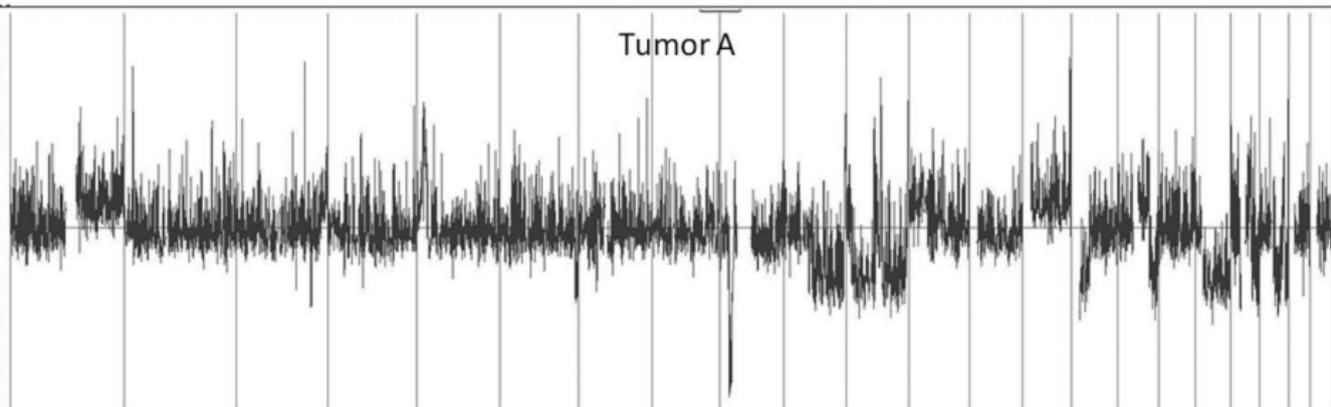
Tumor B



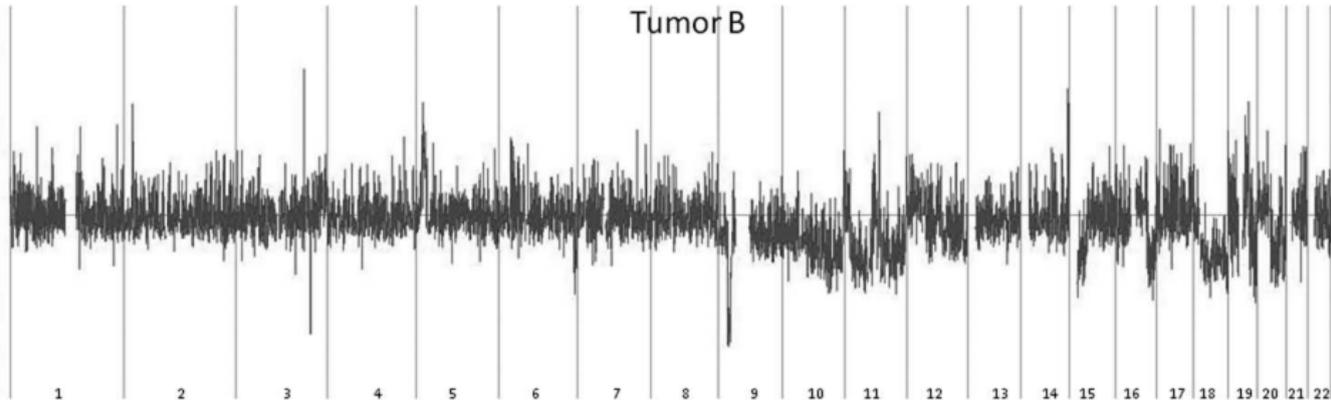
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Case 2

Tumor A



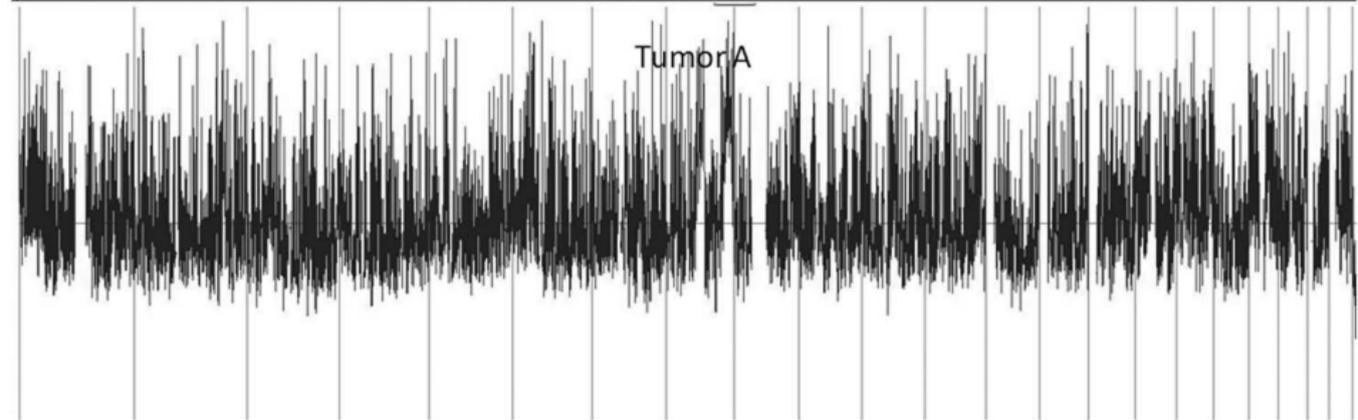
Tumor B



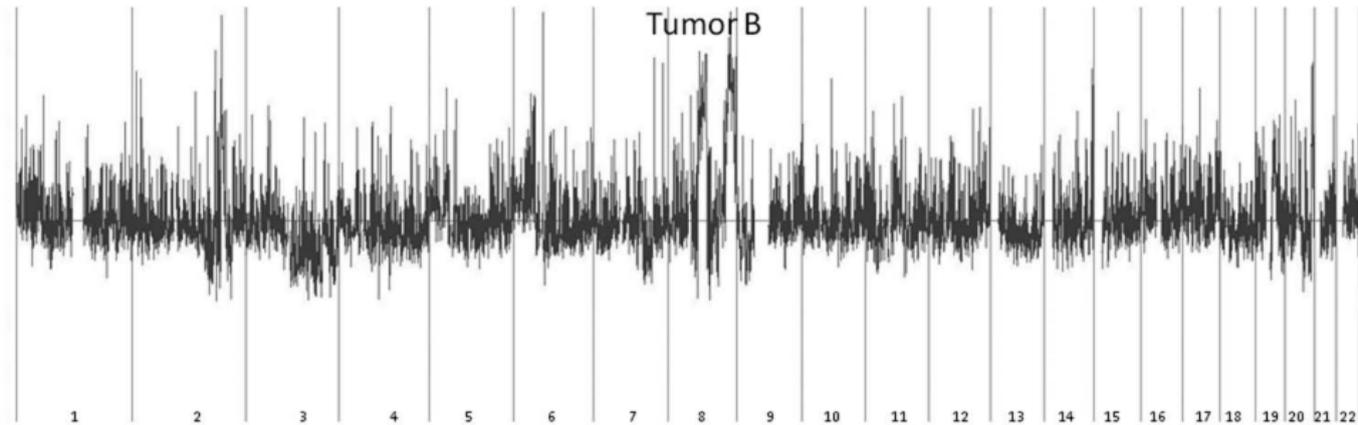
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Case 3

Tumor A



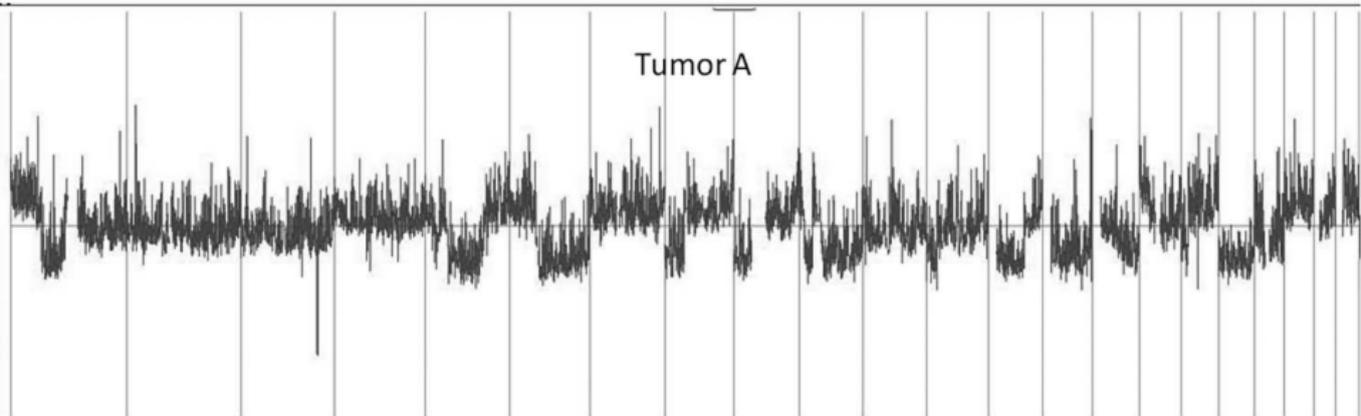
Tumor B



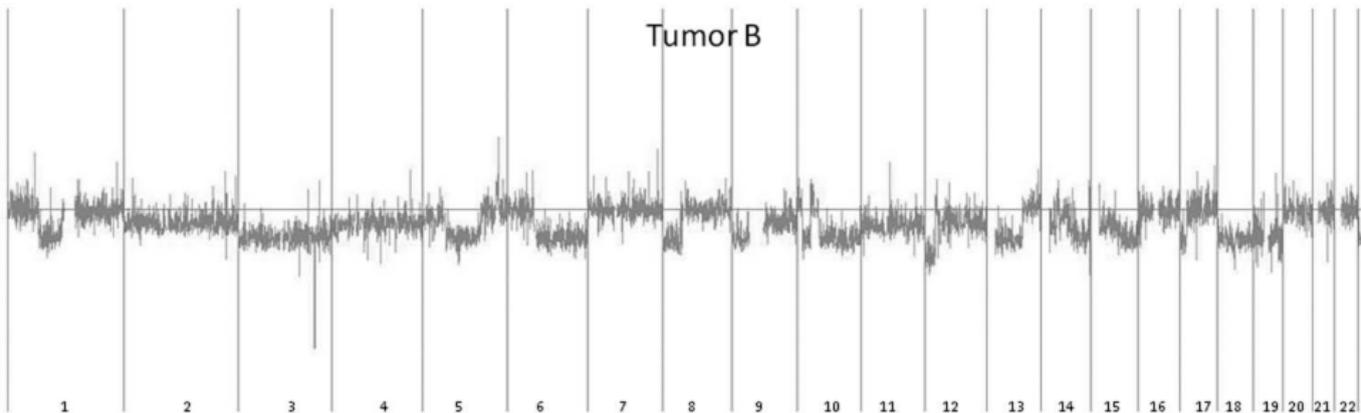
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Case 4

Tumor A



Tumor B



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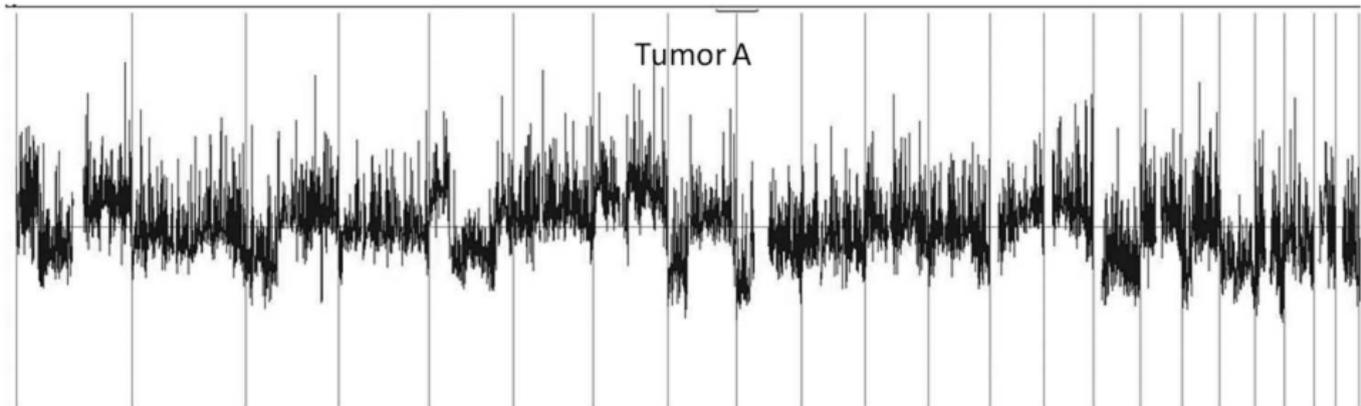
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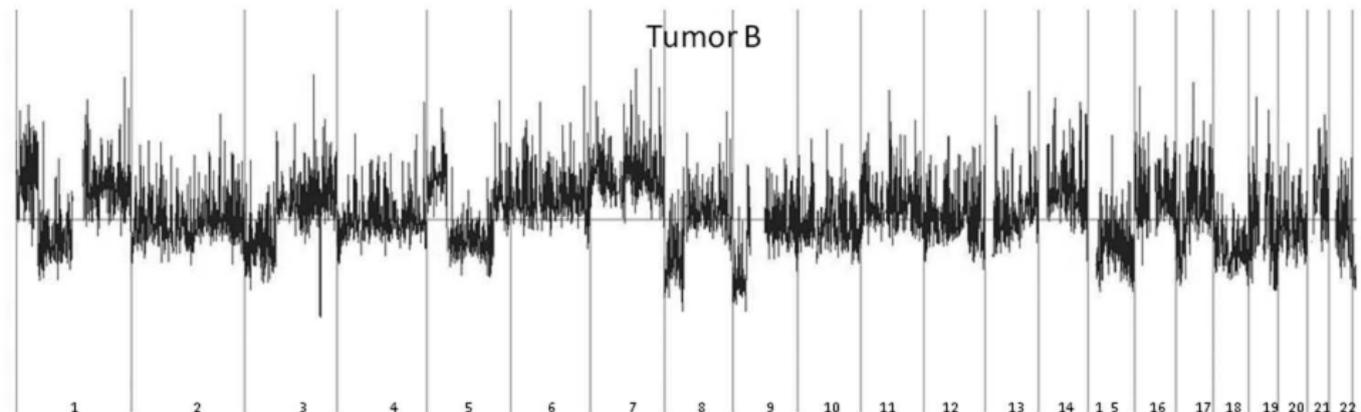
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Case 5

Tumor A



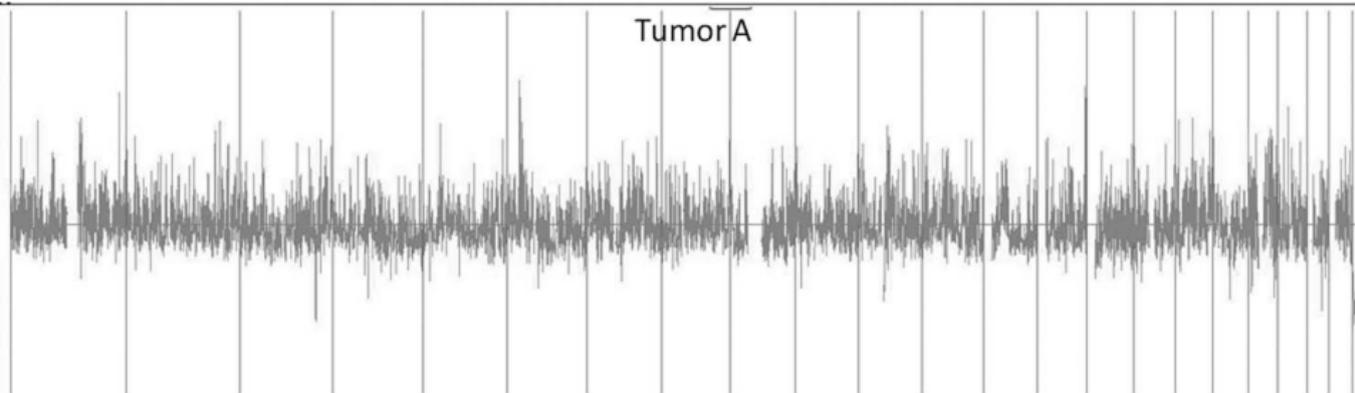
Tumor B



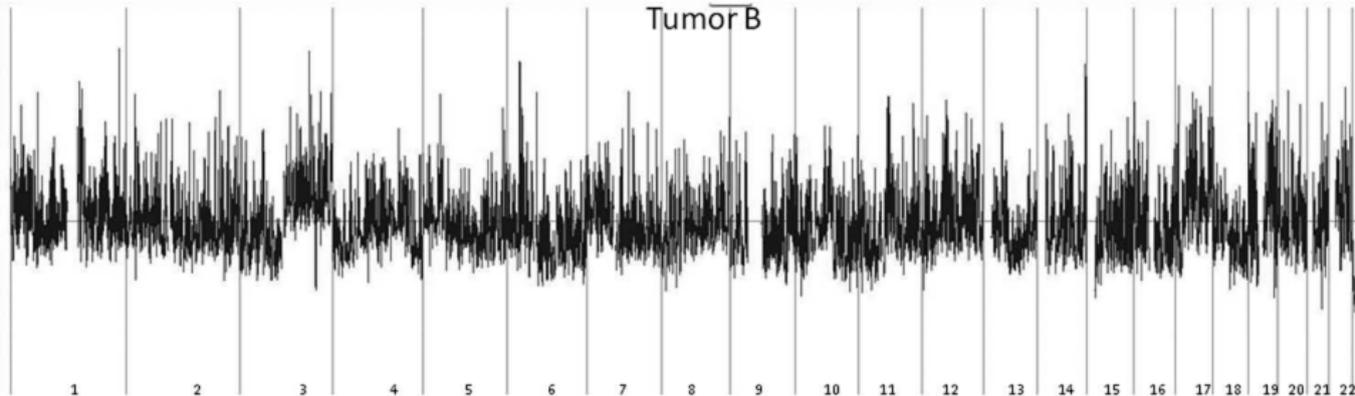
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Case 6

Tumor A



Tumor B



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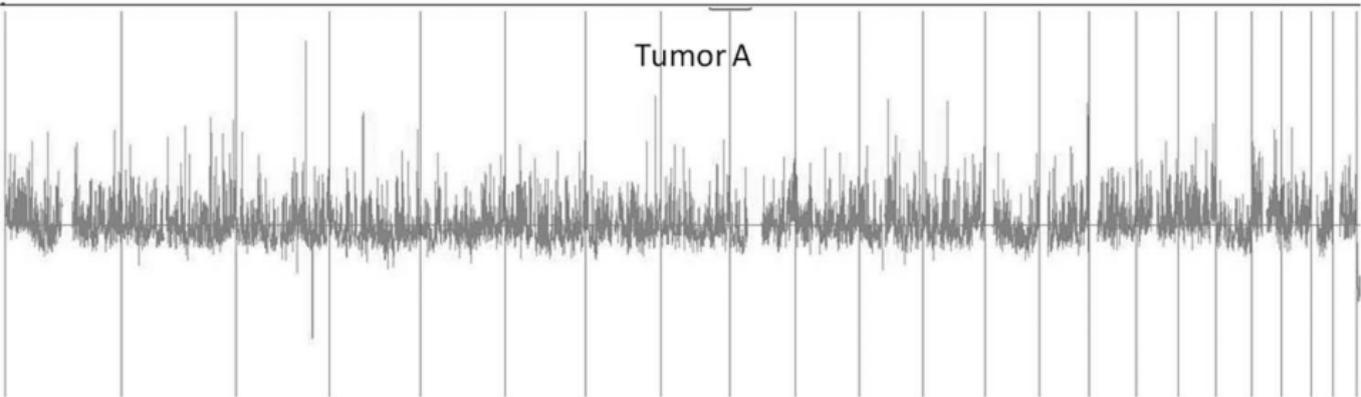
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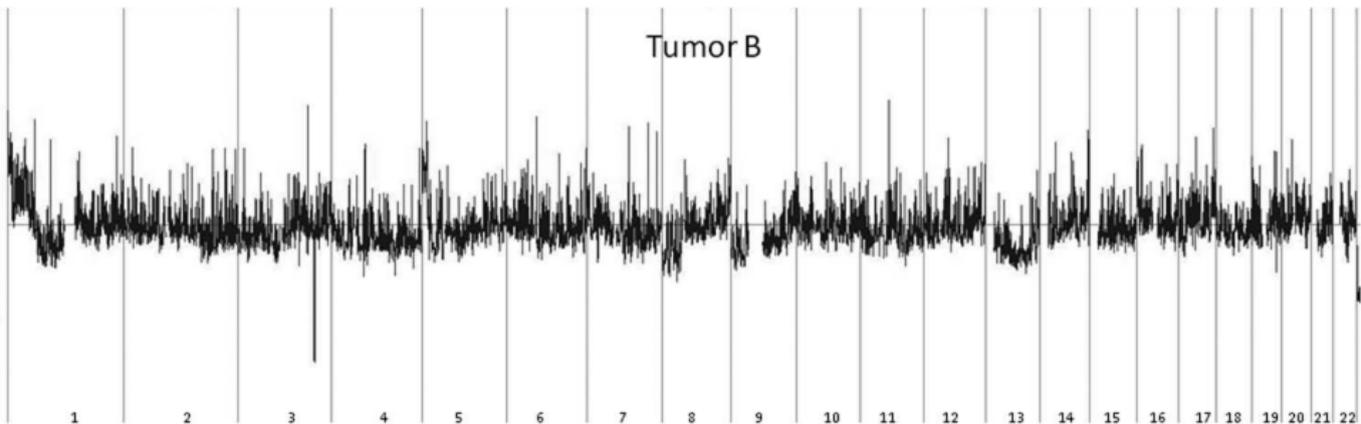
22

Case 7

Tumor A



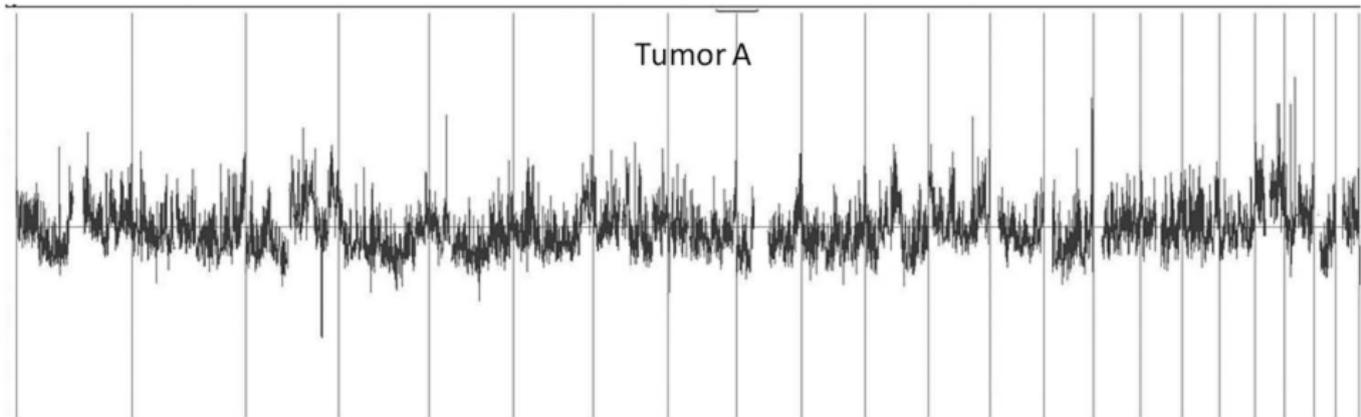
Tumor B



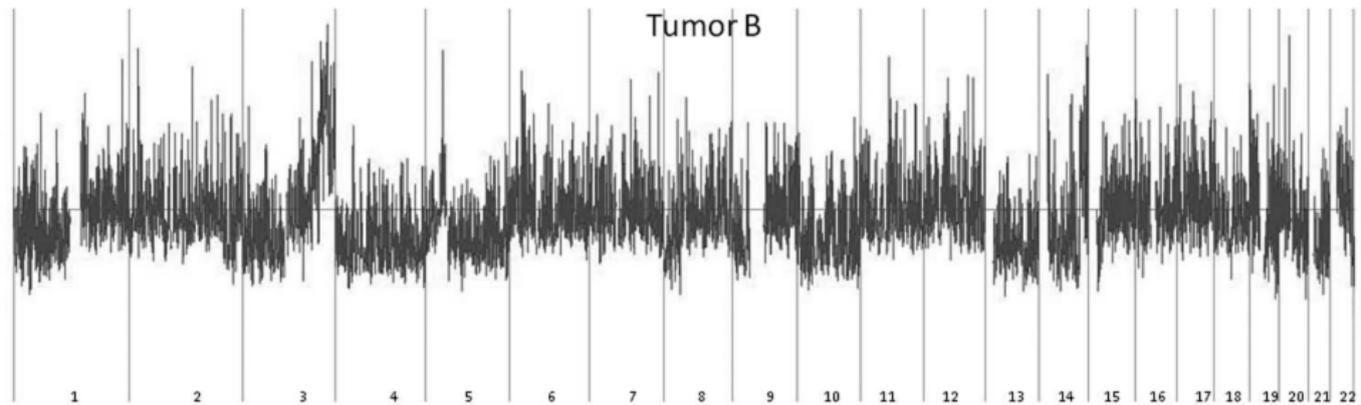
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Case 8

Tumor A



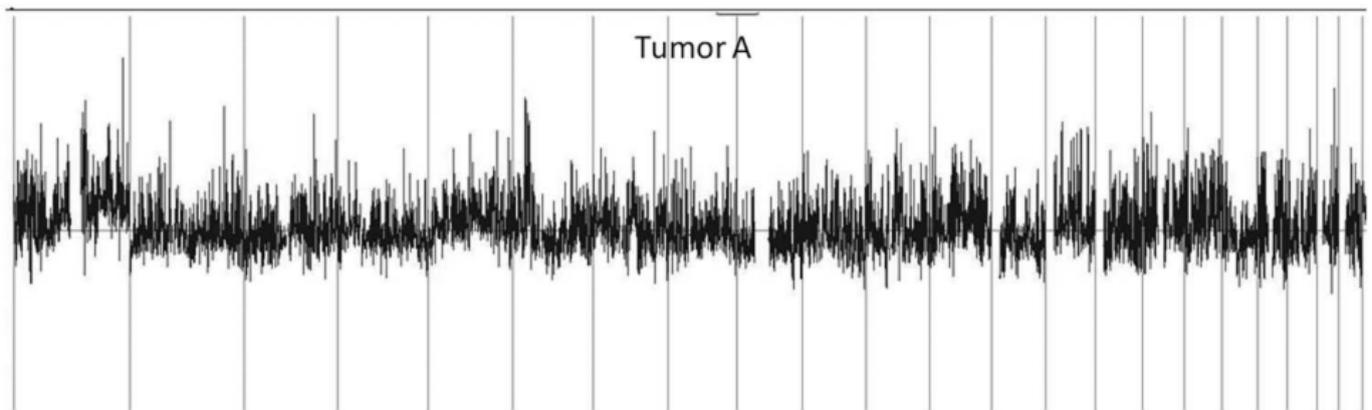
Tumor B



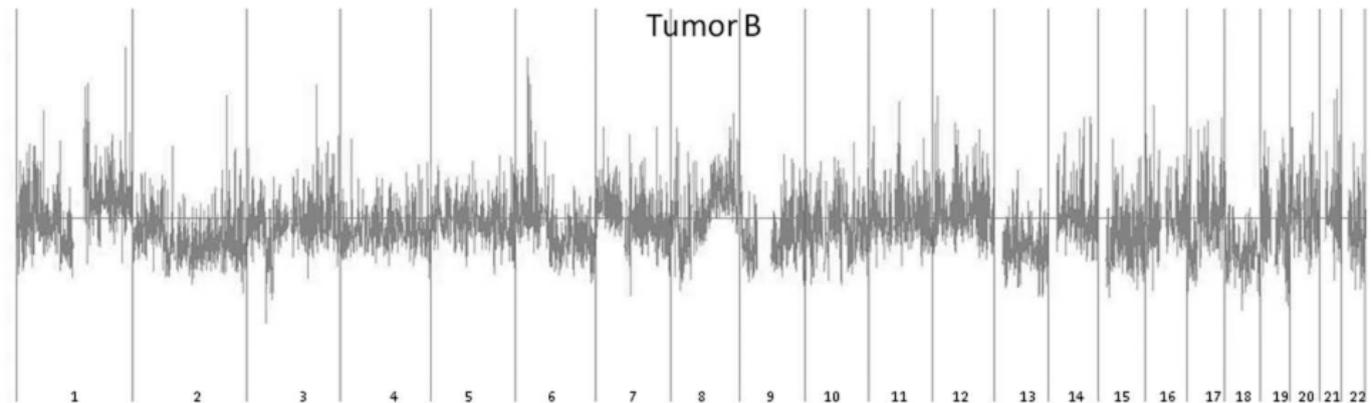
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Case 9

Tumor A



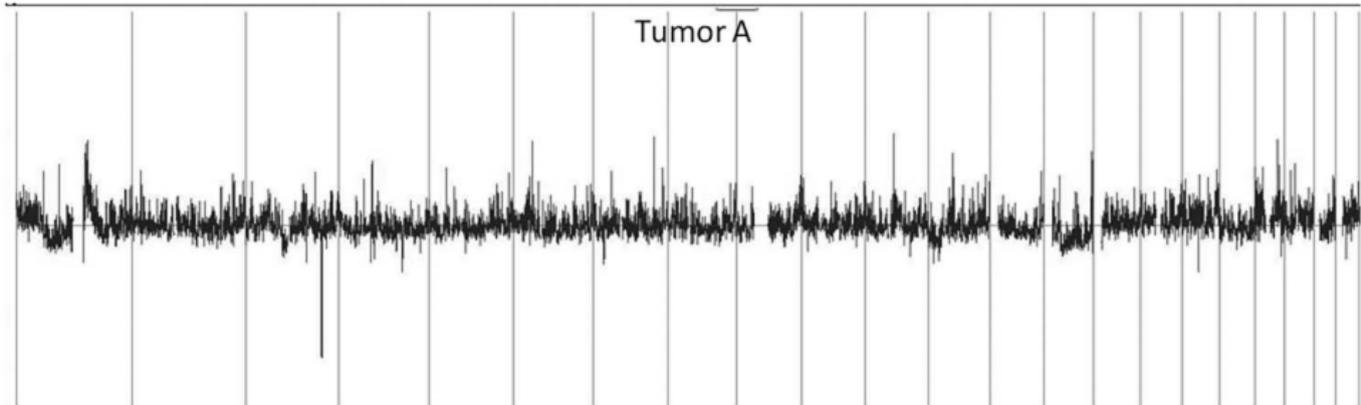
Tumor B



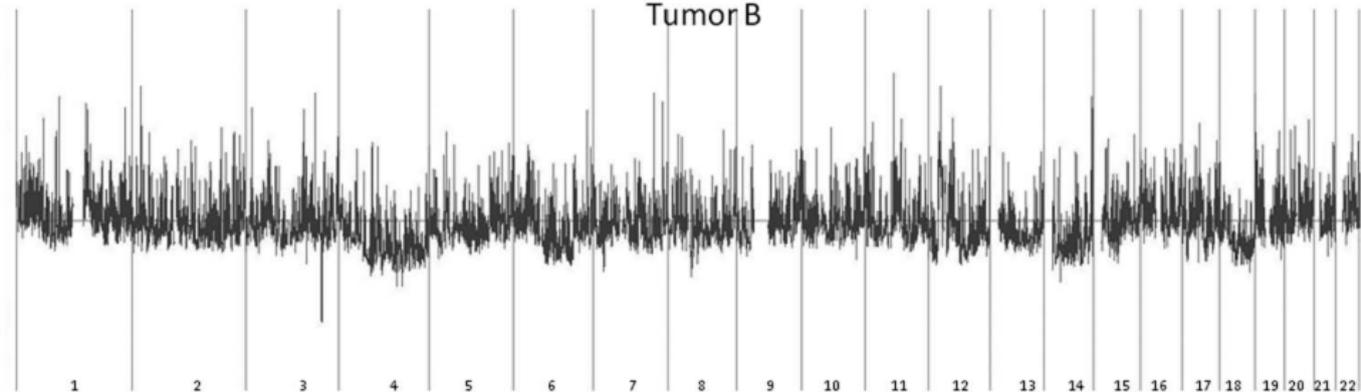
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Case 10

Tumor A



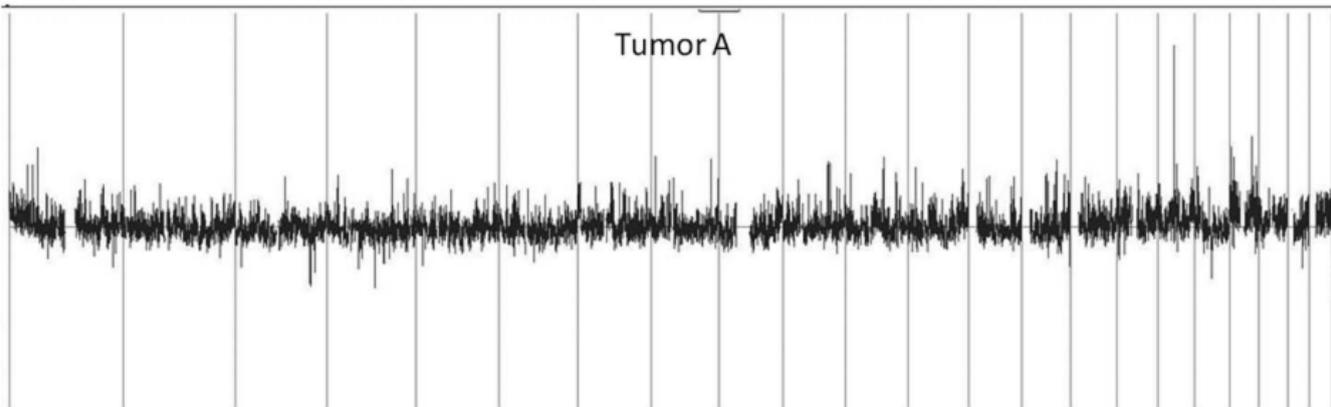
Tumor B



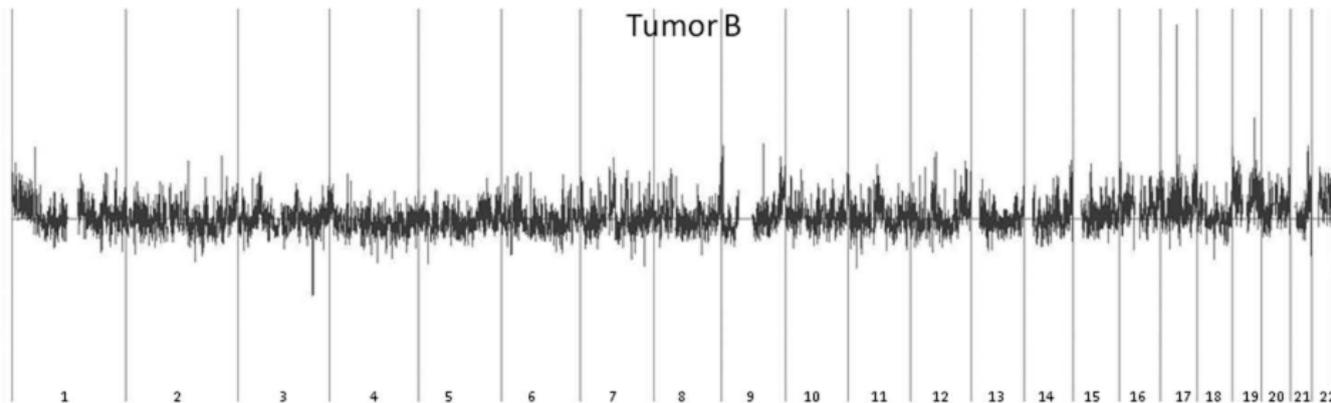
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Case 11

Tumor A



Tumor B



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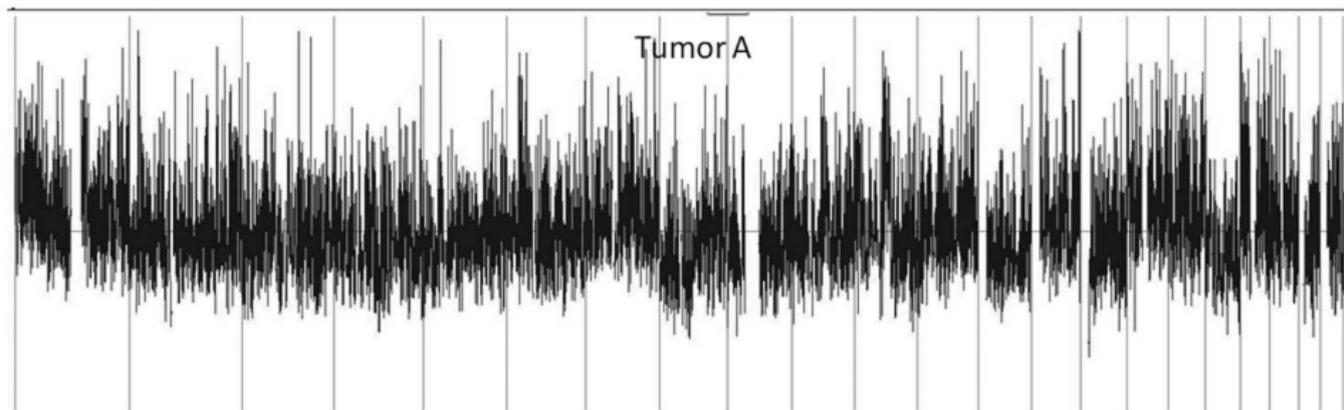
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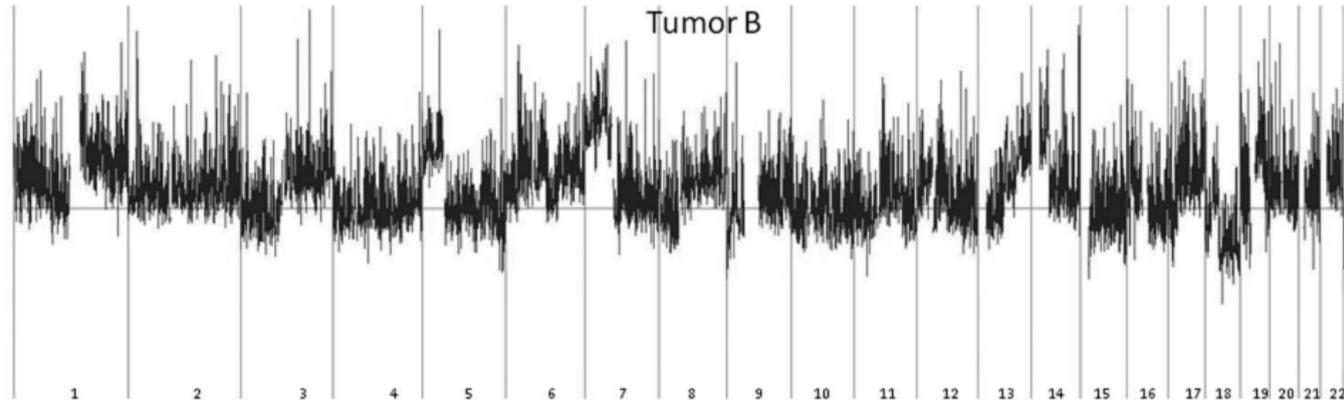
22

Case 12

Tumor A



Tumor B



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22