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Original Article

Screening of the copy number increase of *AKT* in lung carcinoma by custom-designed MLPA

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Abstract: Treatments for lung cancer include therapies targeting aberrant oncoproteins, but there remains a high medical need for novel therapies. Our previous studies showed that gene amplification/high-level polysomy of *AKT1/2* occurs in more than 10% of lung carcinomas. Here, we describe multiplex ligation-dependent probe amplification analysis (MLPA) as a high-throughput method to evaluate copy number increases (CNIs) of *AKT1/2* in lung carcinomas. The performance of MLPA using custom-made probes in formalin-fixed paraffin-embedded tissue was evaluated by comparing it to immunohistochemistry and fluorescence in situ hybridization analysis (FISH). By MLPA, we found 4 out of 30 samples harboring gene "gain" when the conventional cutoff value (> 1.3) was used. Two samples with gene amplification by FISH had MLPA values of 1.85 and 1.75, which were lower than the conventional cutoff for "amplification" (> 2.0). Moreover, samples with CNIs due to polysomy by FISH gave MLPA values between 1.13 and 1.47, so some samples had lower values than 1.3. The reasons appeared to be stromal contamination and the presence of carcinoma cells without CNIs. However, when we changed the cutoff for "gain" to the "average+2xstandard error", we detected CNIs in 10 samples, with only one each of false-positive and false-negative results. The sensitivity was 90% and the specificity was 98%. Consistently, all cases exhibiting CNI by this criteria revealed Akt activation. In conclusion, MLPA implemented with custom-made probes and an optimized cut-off value is a feasible screening method to semi-quantitatively detect oncogene aberrations, and may contribute to the design of individualized, molecularly targeted therapies against lung carcinoma.

Keywords: *AKT,* gene increase, lung cancer, FISH, MLPA

Introduction

One of the major current issues of cancer precision medicine is how to determine the most appropriate treatment based on the genetic makeup of individual tumors. Lung cancer is one of the most common malignancies and ranks the first among all cancer deaths worldwide [1]. Despite advances in genomic analysis and the clinical success of some molecularly targeted therapies, there remains a significant clinical need to identify additional therapeutic targets for the large number of patients with advanced carcinomas. In addition, many cancers are characterized by genetic heterogeneity, which can allow the cancer to evade the effects of therapies directed at a single molecular target. Thus, a semi-quantitative analysis to determine the prevalence of the specific targeted gene is an important component of this therapeutic approach.

Currently, immunohistochemistry (IHC) is the most common method used by pathologists to assess protein overexpression and activation in cancers and for particular genes, such as EGFR and HER2, and a good correlation is observed between protein overexpression and gene amplification [2, 3]. For gene amplification, the most common method used for detection is fluorescence *in situ* hybridization (FISH) [4]. However, widespread use of this method has been limited due to the cost, the technical difficulties and the complexity of analysis resulting

from intratumoral heterogeneity [5-7]. Therefore, there is a great clinical need to develop cost-effective and high-throughput alternative approaches to detect copy number increases (CNIs) in cancer samples. One potential method is multiplex ligation-dependent probe amplification (MLPA), a polymerase chain reaction (PCR) -based high-resolution method that allows copy number detection of up to 40 target genes with amplicon lengths of up to 500 nucleic acids in one reaction [8-10] and requires only small amounts (50 ng) of DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues [11-14]. Therefore, compared to FISH, MLPA has potential as a more inexpensive and practical method for determining CNI in the pathology laboratory.

Akt1-3 are 56 kDa proteins encoded by the *AKT1-3* genes located at the genetic loci 14q32, 19q13 and 1q44, respectively. Akt1-3 genes provide effector functions downstream of the phosphoinositide 3-kinase (PI3K) cascade and have been extensively investigated for their key roles in a number of cancers [15, 16]. Their tumor-promoting properties can arise from aberrant upstream growth factor signaling, or as a result of gene amplification of *AKT1 or AKT2*, which is observed in 3% to 6% of solid tumors, including lung carcinomas [17]. In our previous study, we observed protein activation of Akt1/Akt2 concomitant with CNIs in *AKT1* and *AKT2* due to amplification and high-level polysomy in 12.6% and 14.8%, respectively. These increases were correlated with negative clinicopathological profiles, suggesting that Akt1/2 may be promising candidate targets [17]. These results prompted us to explore potential methodologies to precisely and conveniently evaluate the CNIs in the AKTs and their correlations with overexpression/activation of the Akt proteins, including samples with intratumoral heterogeneity.

As the extended study of our previous work aiming to establish a high-throughput scheme, we conducted several screens. The first used IHC to assess the overexpression of total-Akt (T-Akt), phosphorylated Akt (p-Akt), Akt1 and Akt2. The second used MLPA with custom-made probes and DNA from the whole area of one representative section on glass slides of surgical specimens to quantitatively evaluate CNIs in *AKT1* and *AKT2*. The third used FISH with established probes to look at CNIs in *AKT1* and *AKT2*. These analyses were combined in our assessment of the samples, the results among the assays were compared, and finally the usefulness and robustness of each method were characterized.

Materials and methods

Patients

Tissue samples from a total of 30 cases of lung carcinoma which had been obtained at surgery in the Department of Thoracic Surgery, Jichi Medical University Hospital between 2014 and 2017 were used for this study. These included 17 cases of adenocarcinoma (AC), 10 cases of squamous cell carcinomas (SCC) and 3 cases of small cell carcinoma (SmCC). Additionally, adjacent non-neoplastic tissues (NT) were also obtained in 10 out of these 30 cases (4 cases of AC, 5 SCC and 1 case of SmCC). Their clinicopathological profiles are summarized in **Table 1**. None of the patients had received preoperative chemotherapy. This study was approved by the Institutional Ethical Review Board (approval No. 17-45) and written informed consent was obtained from all patients.

Immunohistochemistry (IHC)

Surgical specimens were fixed in 10% buffered formalin and embedded in paraffin. The expressions of total-Akt (T-Akt), phosphorylated-Akt (p-Akt^{Ser473}), Akt1, and Akt2 were evaluated by IHC. After heat activation, primary antibodies were applied as follows: total-Akt (T-Akt, polyclonal, Cell Signaling Technology [CST], Beverly, MA) 1:300; Akt1 (monoclonal, C73H10, CST) 1:50, Akt2 (polyclonal, Abcam, Cambridge, UK) 1:150, phosphorylated-Akt (p-Akt^{Ser473}, monoclonal, D9E, CST) 1:50. The sensitivity and the specificity of the antibodies had been previously validated [17-19]. Visualization was performed with a CSAII kit (Catalyzed Signal Amplification System 2, Dako, Glostrup, Denmark).

IHC expression was evaluated by two observers (YD and AO) and the IHC scores were determined semiquantitatively by multiplying the "positive fraction" by the "intensity score" according to the following tier system: (i) the "positive fraction" (labeling index) was categorized as negative (0), < 10%; 1+, ≥ 10%, < 50%; 2+, ≥ 50%, (ii) the "intensity-score" was categorized as 0, no staining; 1, the same or weaker than non-neoplastic cells; 2, more intense than non-

Table 1. Patients and tumor characteristics

Characteristics		Number (30 cases)
Gender	Male	18
	Female	12
Histology	AC[2)]	17
	SCC[3)]	10
	SmCC[4)]	3
T factor (NSCLC[1)]) 27 cases	T1	11
	1mi	2
	1a	3
	1b	3
	1c	3
	T2	13
	2a	8
	2b	5
	T3	3
N factor (NSCLC) 27 cases	N0	16
	N1	8
	N2	3
Stage (NSCLC) 27 cases	I	15
	IA1	4
	IA2	2
	IA3	2
	1B	7
	II	8
	IIA	1
	IIB	7
	IIIa	4

1) NSCLC, non-small cell carcinoma; 2) AC, adenocarcinoma; 3) SCC, squamous cell carcinoma; 4) SmCC, small cell carcinoma.

neoplastic cells. The positive staining intensity in non-neoplastic tissue was arbitrarily defined as 1. Thus, each case could be given a score of 0, 1, 2, or 4. Since, in our previous analysis, none of the IHC negative cases exhibited gene amplification or high-level polysomy, we regarded a score > 0 as "positive" [17]. Discordance was resolved by discussion.

Multiplex ligation-dependent probe amplification (MLPA)

Preparation of DNA: After IHC staining, high-molecular weight DNA was manually extracted from the adjacent 6-μm-thick sections containing the same areas that were also used for FISH, according to the protocol as previously described [10, 13]. At least 1 μg of sample DNA with an OD_{260}:OD_{280} ratio within 1.1-1.7 was prepared from each sample [13].

Probe design: Gene sequences were retrieved from the NCBI Gene Bank (Reference Sequence: NG_012188.1 for *AKT1*, NC_000019.10 for *AKT2*). To avoid amplification artefacts, 7 pairs of custom-made probes for each target gene were designed to generate different lengths of the final product, according to the "guidelines for probe design" by MRC-Holland (Amsterdam, the Netherlands) (**Table 2**). This enabled the simultaneous analysis of 7 sets of probes in a single reaction. Each probe consists of two components: sequences complementary to the 5' or 3' target sequence, predominantly in exons, and a non-hybridizing tail containing a universal primer binding site [8, 20] (**Figure 1**). These two fragments are joined by a DNA ligase in the initial step of the MLPA reaction, subsequently forming a single amplifiable template whose length is defined by the individual probes. As a result, the shortest probe was 94 nucleotides (nts) in length, including the primer biding site, while the longest probe length was 148 nts. The difference among the probe lengths was 4 to 15 nts (**Table 2**). All the probes were synthesized at the 100nmol scale and purified by high performance liquid chromatography by Integrated DNA Technologies Co. Ltd. (Skokie, Il.).

MLPA reaction: Each experimental procedure was performed as previously described [10, 11]. In brief, 7 sets of probes and genomic DNA from samples (100 ng) were mixed with an MLPA reagent (MRC-Holland) except probemix, and with a pre-made reference mix (SALSA MLPA P200 Human reference probemix, MRC-Holland), including 14 kinds of control DNA [20]. PCR reactions were performed and the resulting MLPA PCR products were separated and analyzed as described [11, 21]. Genemarker software was used to analyze the peak areas of the MLPA PCR products, and the ratios were normalized to a healthy control. The test was duplicated and the mean value of all the probe peaks was calculated. Initially, peak values below 0.7 were defined as copy number "loss", between 0.7 and 1.3 as "normal", between 1.3 and 2.0 as "gain", and > 2.0 as "amplified", as previously established [5, 6, 22, 23].

Fluorescence in situ hybridization (FISH)

The FISH analysis was conducted on whole sections adjacent to those used for IHC staining. Bacterial artificial chromosome clones were used for the *AKT1* and *AKT2* probes [17]. The

Table 2. Oligonucleotide Custom-made Probes for the MLPA assay

Name		Length[3]	Total length[4]
AKT1-1F[1]	GGGTTCCCTAAGGGTTGGACCCAGGCAGCCCCTTTGACTTCTTTG	45	94
AKT1-1R[2]	ACCCAGGCTGGCTCGGCCTTCCCTAATCTAGATTGGATCTTGCTGGCAC	49	
AKT1-2F	GGGTTCCCTAAGGGTTGGACCTGACGCTCCTCGAACATGAATAGAATGTG	50	103
AKT1-2R	GAGACCACAACCCCCACACATGTCGTTGGTTCTAGATTGGATCTTGCTGGCAC	53	
AKT1-3F	GGGTTCCCTAAGGGTTGGACTACTAACCTCGTTTGTGCAGCCAACCCTCCTTCA	54	109
AKT1-3R	CAATAGCCACGTCGCTCATGGTGCCCGAGGCTTCTAGATTGGATCTTGCT GGCAC	55	
AKT1-4F	GGGTTCCCTAAGGGTTGGACGACAAGTCCGTCAGTGAGGAGCACCCAGT CCAGGGTGGTTA	61	125
AKT1-4R	CAGACCCATAATTACAGCAGTCGGGAGGCAGCAAGTAAGTTTCTAGATTG GATCTTGCTGGCAC	64	
AKT1-5F	GGGTTCCCTAAGGGTTGGACTGCCCGCTCTGTGGGAAGACCTTCTCGTGCATCAACACACTGAAGA	66	133
AKT1-5R	GGCACGAGCGGACACACTCGGGTGAGAAGCCCTATACGTGTGTTTCTAGAT TGGATCTTGCTGGCAC	67	
AKT1-6F	GGGTTCCCTAAGGGTTGGACCCAAGCTCCCCCCGTTTGGGGTCAAGGCTG CCCTCCCTCCTCGAGCAT	68	141
AKT1-6R	CTGGGCCTCCTCCCTGCCAGGTCCCAGAAGAGTCAGACCAGGCCCTGGAT TCTAGATTGGATCTTGCTGGCAC	73	
AKT1-7F	GGGTTCCCTAAGGGTTGGACTGTGAGCGCCGTTTCACGCAGTCCGGGGAC CTCTACCGCCACGTCCGCAAGT	72	146
AKT1-7R	TTCACTGTGGCCTCGTCAAGTCCCTTCTGGTGTGATGCATCCCTGTGGGTT TCTAGATTGGATCTTGCTGGCAC	74	
AKT2-1F	GGGTTCCCTAAGGGTTGGACGCTTGTGGAGCCAGCCTTCTTTGATG	46	97
AKT2-1R	ACAGACACCTCATTCATGGTGGCAGCGTTCTAGATTGGATCTTGCTGGCAC	51	
AKT2-2F	GGGTTCCCTAAGGGTTGGACTAAGTTCAAACACCCTTCTTGGGAAAAGCTC TAC	54	112
AKT2-2R	AGGATAACCACACATGCTCAACTACGGGGCCAGCTTCTAGATTGGATCTTG CTGGCAC	58	
AKT2-3F	GGGTTCCCTAAGGGTTGGACTGCTACGGAGAAGTTGTTTAAGGGGGGTA GAGTCTGA	57	118
AKT2-3R	TCAGGGGCCTCGGGCCTCTCCTTGTACCCAATGAAGGATCTAGATTGGAT CTTGCTGGCAC	61	
AKT2-4F	GGGTTCCCTAAGGGTTGGACTAAAGGGCAGTGACTAGGGGAGGGCTGAC CCCAAGCTGAACA	62	127
AKT2-4R	GGGTTCTAACCAAACGCTCAGGAGCTCCCAGGTGACAGCAACTCTAGATT GGATCTTGCTGGCAC	65	
AKT2-5F	GGGTTCCCTAAGGGTTGGACCAGACACCTCAGGCGCCAGGTACTCCGGG GTCCCACAGAAGGT	63	135
AKT2-5R	TTTCATGGTGGCCCCGTCACTGATGCCCTCTTTGCAGAGGCCAAAGTCAT CTAGATTGGATCTTGCTGGCAC	72	
AKT2-6F	GGGTTCCCTAAGGGTTGGACTCACAGCGGTCAGGGGGTGTGATTGTGAT GGACTGGGCGGTAAATTC	67	139
AKT2-6R	ATCATCGAAGTACCTTGTGTCGACCTCGGACGTGACCTGAGGTTTGAAGT CTAGATTGGATCTTGCTGGCAC	72	
AKT2-7F	GGGTTCCCTAAGGGTTGGACACAAAAAGAGCAGGAAACTACCAATTTATG ATGCCGTGTCCATTTGCAGAGA	72	148
AKT2-7R	GGTAATCAGCACCAAAATGAGTACTCAAGGCCCTGCGACCTCGGGTGAAT TTCTCTAGATTGGATCTTGCTGGCAC	76	

Abbreviations: 1) F, 5'-half of custom-made probe including primer sequence of 23 nucleotides; 2) R, 3'-half of custom- made probe including primer sequence of 23 nucleotides, 3) Length, the number of nucleotides; 4) Total length, total number of nucleotides, including 5'-, and 3'-half.

reference probe for *AKT1* consisted of the pericentromere region spanning *TEP1* (14q11.2, RP11-203M5), and that for *AKT2* spanned *JAK3* (19p13.11, RP11-124K10) [19]. The probes were labelled with SpectrumOrange™ (for the *AKT* probes) and SpectrumGreen™ (for the reference probes) and analyzed according to previous procedures [10, 11]. Scoring and evaluation of gene increases were performed by two observers (YD and AO) who manually counted the *AKT1* and *AKT2* signals, as well as the reference genes in at least 50 tumor cell nuclei. All FISH analyses were performed blind to the results of the IHC and MLPA analyses. Results were classified into 4 strata: 1) disomy (≤ 2 copies in > 90% of the cancer cells); 2) low-level polysomy (≥ 3 copies of the target accompanied by the same number of reference genes in ≥ 10%~< 40% of the cells, without amplification); 3) high-level polysomy (polysomy in ≥ 40% of the cells, without amplification); and 4) amplification (the presence of tight clusters, average target/reference gene ratio of > 2) [10, 17, 24, 25]. The fraction (%) of carcinoma cells harboring *AKT* CNIs on the section was also evaluated. Tumors in which fewer than 50% of the tumor cells exhibited CNIs were arbitrarily defined as tumors with a "heterogeneous gene increase" [11, 26].

Comparative analysis of IHC, FISH and MLPA

IHC scores, the pattern of CNIs, the fraction of carcinoma cells with CNIs by FISH for *AKT1/2* and the values obtained by MLPA were compared, and the results of each analysis were validated.

Results

IHC analysis

The results of the IHC analysis are presented in **Figures 2**-**4** and summarized in **Table 3**. In T-Akt staining, IHC revealed variable cytoplasmic and

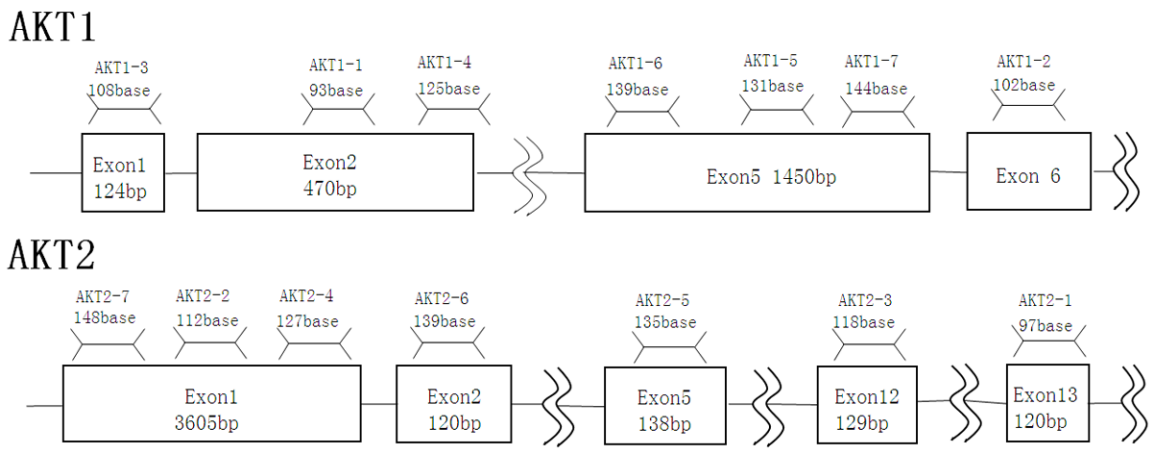

Figure 1. The complementary locations and the lengths of 7 probes recognizing *AKT1* (AKT1-1~7) and *AKT2* (*AKT2*-1~7).

occasional nuclear staining at various intensities, depending on the region. Positive IHC scores for T-Akt were observed in 27 cases of carcinoma (6 cases of score 4, 12 of score 2 and 9 of score 1). In contrast, the 10 non-neoplastic samples had a score of either 1 (6 cases) or 0 (4 cases). p-Akt staining of tumor samples gave a positive score in 22 cases and negative in 8 cases. Akt1 staining gave a positive score in 19 cases, and Akt2 in 21 cases. Among the non-neoplastic samples, none gave a score of greater than 1.

MLPA analysis

MLPA analysis was successfully performed on all 40 FFPE tissue samples. The mean MLPA peak values are shown in **Table 3** with the results of the FISH analyses and IHC scores. The status of the *AKT1/2* genes of the 30 tumors was determined according to the criteria originally defined in Materials and Methods. Two samples of "gain" for *AKT2* (cases 1 and 2) and two for *AKT1* (cases 18 and 28) were found with MLPA values from 1.39 to 1.85. For both *AKT1* and *AKT2,* twenty-eight (28) samples were scored as "normal" with MLPA values from 0.82 to 1.28. No cases of "amplification" or "loss" were found in either *AKT1* or *AKT2*. All samples from non-neoplastic tissue had 'normal' MLPA values for *AKT1* and *AKT2*, ranging from 0.86 to 1.04.

The number of samples categorized as "gain" was much smaller than expected from our previous results, where amplification/high-level-polysomy of *AKT1* and *AKT2* was found in more than 10% of the total cases [17]. Therefore, we reset the criteria of MLPA to define "gain" as the "mean MLPA value + 2x standard error (SE)" of the 30 tumor samples: this reset the cutoff for *AKT1* at 1.131 (1.059 + 0.036 × 2), and that for *AKT2* at 1.110 (1.040 + 0.035 × 2). By this re-calibrated cutoff, 4 samples in *AKT1* and 6 samples in *AKT2* were now categorized as "gain".

FISH analysis

The results of the CNI by FISH and the proportion of carcinoma cells having CNIs are shown in **Table 3**. CNIs of *AKT1/2* were found in 10 samples from 9 cases. These included 2 samples exhibiting gene amplification, 3 samples with high-level polysomy, and 5 samples with low-level polysomy.

Among the 2 samples exhibiting amplification, one (case 1) displayed clustered-type *AKT2*-amplification in 53% of the carcinoma cells (**Figure 2**). In another sample (case 18), CNIs were observed as more than 4 *AKT1* signals, while the reference signals were normal, giving a target/reference ratio of > 2.0. *AKT1*-amplified cells comprised 55% of the carcinoma cells in the section examined. Among other 3 samples with *AKT1* CNIs, one sample showed high-level polysomy in 64% of the cancer cells examined (**Figure 3**, Case 28), and 2 samples exhibited low-level polysomy of chr.14 (Cases

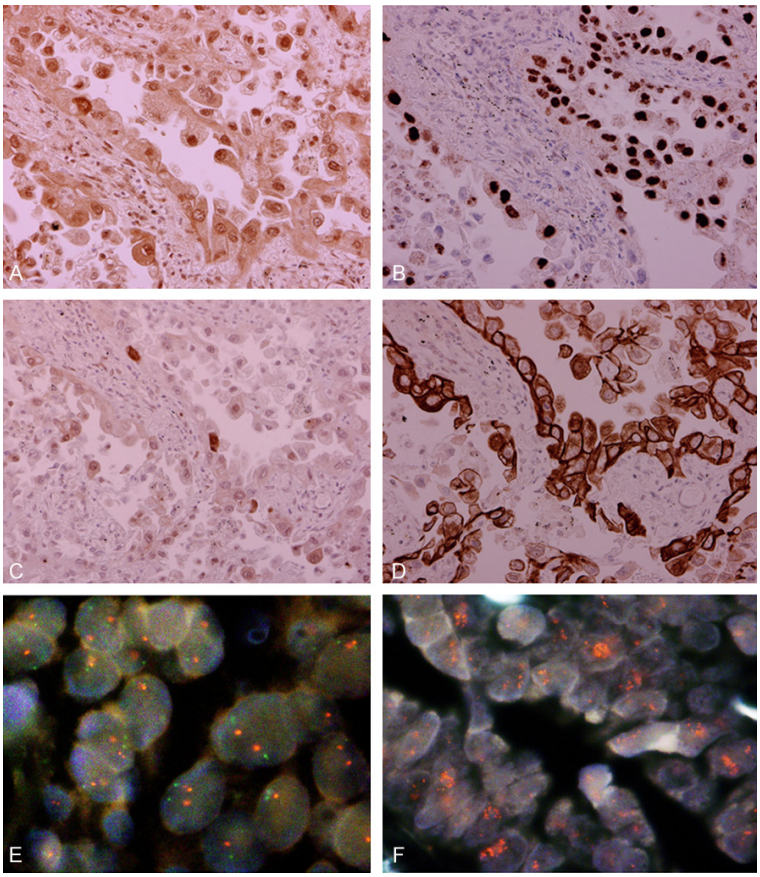

Figure 2. Results of immunohistochemical staining and fluorescence *in situ* hybridization analysis (FISH). A case of adenocarcinoma (Case 1) that exhibited nuclear/cytoplasmic positive staining for total-Akt (A) and Akt2 (D), nuclear staining for phosphorylated-Akt (B) and Akt1 (C). FISH revealed 2 copies of gene-specific signals (orange fluorescence) and reference probe signals (green fluorescence), indicating disomy of *AKT1* (E) and a clustered-type increase in gene-specific signals, indicating amplification of *AKT2* (F).

3. 4 with 36% and 28%) where *AKT1* is located. The remaining 26 samples exhibited disomy (**Figure 4**). Analysis of *AKT2* revealed 2 cases with high-level polysomy of chr.19 (found in 81% of the cancer cells in case 2, and 43% in case 19), and 3 samples with low-level polysomy (found in 17%, 38% and 22% of the cancer cells in cases 3, 20 and 29, respectively). The remaining 24 samples exhibited disomy (**Figure 4**). No co-amplification of *AKT1* and *AKT2* was found, although one case (Case 3) exhibited polysomy of both chromosomes 14 and 19. This suggests that CNIs may arise in the two *AKT* genes in a mutually exclusive manner.

Collectively, 6 out of 10 samples in which CNIs were detected exhibited intratumoral heterogeneity (< 50%), and the *AKT* CNI-positive and -negative cells were randomly intermingled, even in the single cancer nests.

Correlations among IHC, MLPA and FISH

Comparisons among the results of the IHC, MLPA, and FISH analyses revealed the following (**Table 4**).

IHC vs MLPA: The IHC analysis produced a positive T-Akt score in 27 carcinoma samples and a positive p-Akt score in 22 samples, whereas the MLPA analysis found a net "gain" in the *AKT1* or *AKT2* gene in only 10 samples (from 9 cases), even after optimization of the cutoff values. Thus, the sensitivity of IHC was 100%, and the specificity was 12% for T-Akt, and 32% for p-Akt (**Table 4A**, **4B**). IHC for the individual Akt1 and Akt2 proteins revealed positive staining in 19 and 21 samples, respectively, whereas by MLPA, only 4 and 6 samples scored positive for the "gain" of respective genes. Therefore, even after the optimizing of cutoff values, the concordance between MLPA and IHC was not high, i.e., the sensitivity of IHC was 100%, and the specificity was 42% for Akt1, and 38% for Akt2 (**Table 4C**, **4D**).

IHC vs FISH: 9 of the 27 tumor samples showing T-Akt-overexpression by IHC (4 samples with score 4 and 5 samples with score 2) and 9 of 22 samples showing p-Akt positivity (3 samples with score 4 and 6 samples with score 2) were found to have CNIs as determined by FISH. *AKT1*-CNI was observed in 4 of the 19 Akt1-positive samples, and *AKT2*-CNI in 6 of the 21 Akt2-positive samples. Conversely, all 3 and 8 samples without T-Akt or p-Akt expression, respectively, showed no CNIs of *AKT1* nor *AKT2* (**Table 3**).

MLPA vs FISH: The cutoffs used to designate gene "amplification" by MLPA appeared to be more stringent than for FISH. Two samples that

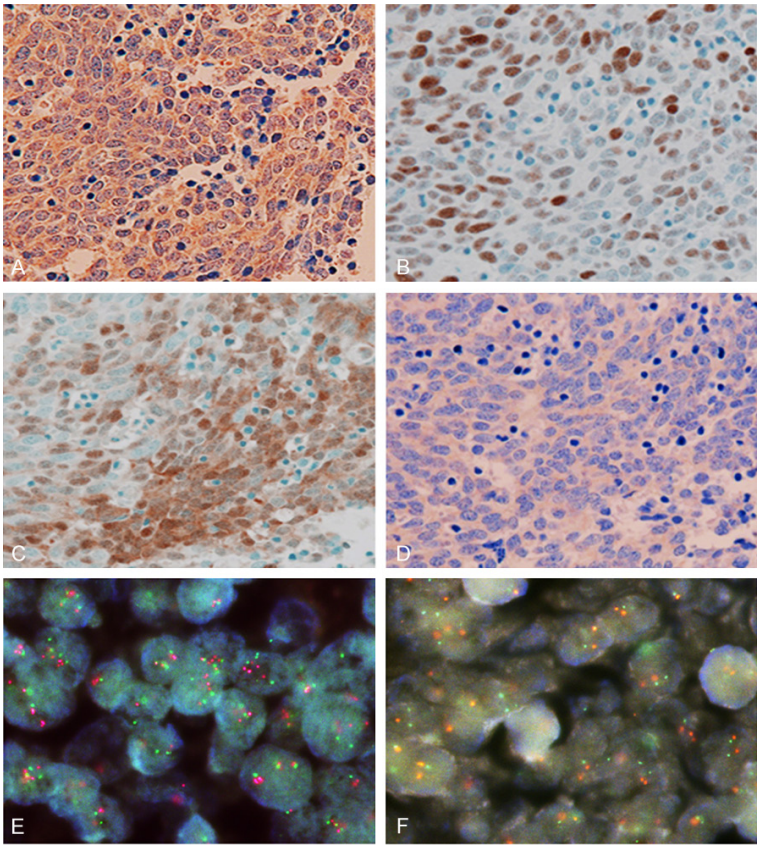

Figure 3. Results of immunohistochemical staining and FISH. A case of small cell carcinoma (Case 28) that exhibited positive cytoplasmic staining for total-Akt (A), nuclear staining for phosphorylated-Akt (B), nuclear/cytoplasmic positive staining for Akt1 (C), and negative staining for Akt2 (D). FISH revealed occasional increases in gene-specific signals (orange fluorescence) with an equal number of reference probe signals (green fluorescence), indicating high-level polysomy of chr. 14 (*AKT1*) (E), but disomy for chr.19 (*AKT2*) (F).

scored positive for "amplification" by FISH had MLPA peak values of 1.85 for *AKT1* (Case 18) and 1.75 for *AKT2* (Case 1). These values would classify these samples as "gain" by MLPA, but not as "amplified", which conventionally has a cutoff value of 2.0. Another sample showed high-level polysomy of chr.14, where *AKT1* is located, in 64% of the carcinoma cells by FISH (Case 28) and had an MLPA value of 1.47, which would classify this sample as exhibiting "gain". Two other samples (Cases 3, 4) showed low-level polysomy in 36% and 28% of the carcinoma cells, and gave MLPA values of 1.28 and 1.13, respectively. These two samples would be classified as "normal" by the standard (conventional) MLPA cutoff value of 1.3. Two cases revealed high level polysomy of chr.19 in 81% (Case 2) and 43% (Case 19) of the carcinoma cells by FISH, but had MLPA values of 1.39 and 1.26, which designated these as "gain" and "normal", respectively. Three samples (Cases 3, 20, 29) revealed low level polysomy in 17%, 38%, and 22% of carcinoma cells but had MLPA values of 1.16, 1.26 and 1.19, respectively, categorizing them as 'normal' by the conventional cutoff. Collectively, among 4 samples categorized as "gain" by the conventional MLPA cutoff, 2 samples showed *AKT*-amplification and the other 2 showed high-level polysomy by FISH. However, when we re-set the MLPA cutoff criteria to "mean MLPA value + 2xSE", an additional 6 samples were re-classified as 'gain', and 5 of these (4 cases) were observed to have polysomy of the chromosome on which the *AKT1* or *AKT2* gene is located (Cases 3 [both *AKT1* and *AKT2*], 19, 20, 29). The sixth sample (case 29) was re-categorized as *AKT1* "gain" with an MLPA score of 1.15 for *AKT1* but was found to be negative for CNI by FISH. Conversely, one sample (Case 4) had an *AKT1* MLPA score of 1.13 and was still scored as "normal" even with the revised cutoff, yet it showed low-level polysomy by FISH.

With the re-optimized MLPA cutoff values, a high level of concordance was found between MLPA and FISH (*AKT1*: 93% [28/30], *AKT2*: 100% [30/30]). In addition, the sensitivity and specificity of 'gain' determined by MLPA versus CNIs detected by FISH was 40% (4/10) and 100% (50/50), respectively, when the conventional cut-off value of 1.3 was used, but was 90% (9/10) and 98% (49/50) when the re-optimized cut-off values were used (**Table 4E**, **4F**).

Discussion

Molecularly targeted therapies require reliable methods for detecting CNIs in order to select

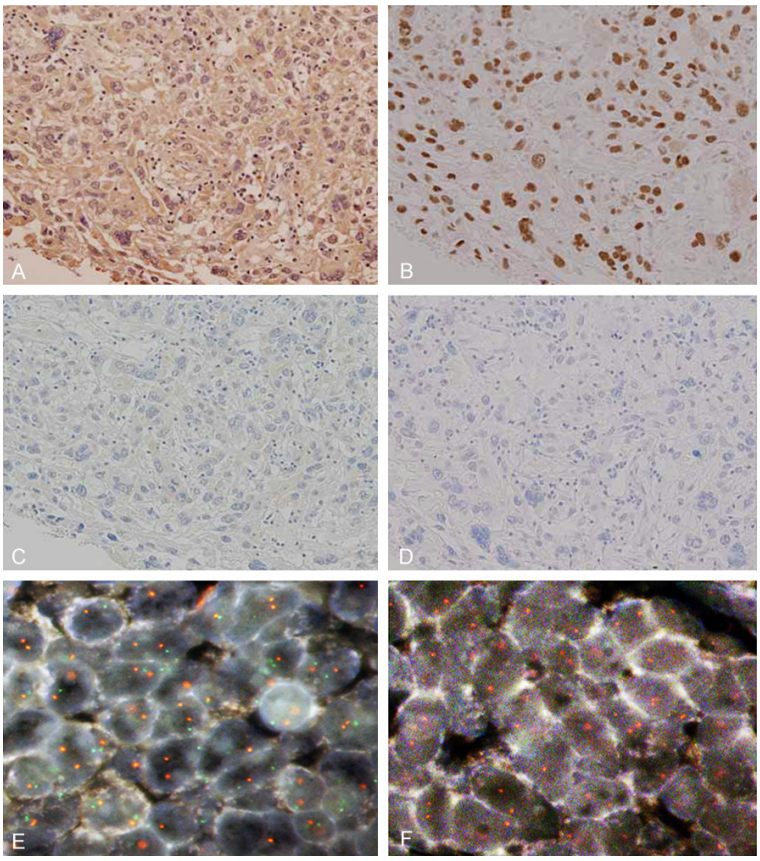

Figure 4. Results of immunohistochemical staining and FISH. A case of squamous cell carcinoma (Case 22) that exhibited nuclear/cytoplasmic staining for total-Akt (A), nuclear staining for phosphorylated-Akt (B) and negative staining for Akt1(C) and Akt2 (D). FISH revealed disomy of both *AKT1* (E) and *AKT2* (F).

the most appropriate therapy for each candidate patient.

In our previous studies, gene amplification/high-level-polysomy of *AKT1* and/or *AKT2* was found in more than 20% of the cases by FISH analysis, and these CNIs were found to correlate with several clinicopathological factors [17]. In the current study, we explored MLPA as a feasible, high-throughput method to detect these CNIs and compared its results with the better-established methods, IHC and FISH.

IHC tends to be the first method of choice in pathology laboratories, and for particular genes, such as EGFR and HER2, a very good concordance has been demonstrated between protein overexpression by IHC and gene increases determined by FISH [2, 3]. However, many of other oncoproteins, including Akt, are often expressed in non-neoplastic tissue at lower levels, as we observed in the present study, and thus, protein overexpression is not always a reliable marker. For such targets, CNIs may be a surrogate marker to detect a responsible or driver gene and IHC is an indirect method for the analysis of possible CNI. Indeed, our attempts to correlate FISH data with IHC have produced variable results. Nevertheless, the overexpression of Akt1 or Akt2 and p-Akt have turned out to be putative markers in the initial screening for the CNIs of the respective genes. Out of 22 cases of carcinoma that scored positive for p-Akt by IHC, 9 cases exhibited CNI of *AKT1* or *AKT2* by FISH. The sensitivity of screening by p-Akt IHC was 100% (9/9), but the specificity was 38.2% (8/21). More specifically, the sensitivity and specificity for Akt1 were 100% (4/4) and 42.3% (11/26), respectively, and those for Akt2 were 100% (6/6) and 37.5% (9/24).

Although the results of MLPA should theoretically be concordant with those of FISH, the MLPA value detected was often lower than that detected by FISH due to contamination of non-neoplastic DNA and of neoplastic DNA without CNIs caused by intratumoral heterogeneity. In the present study, we initially used the conventional cutoff values, prevalently used in recent reports. However, 2 cases classified as 'amplification' by FISH showed a peak MLPA value below the conventional cutoff for "amplification", i.e., 2.0. We presume that this is because the focal distribution and fraction of carcinoma cells harboring the relevant CNIs were low in these samples. A number of samples that scored as 'gain' and even several samples that scored 'normal' by MLPA turned out to harbor focal cell nests with polysomy as analyzed by FISH. In one sample shown to have high-level polysomy by FISH (Case 19), the MLPA value was still within the range considered "normal" (1.26). That sample contained a lower fraction of carcinoma cells harboring relevant CNI

Table 3. Overall results of IHC, MLPA and FISH

Case No.	Histology	IHC score				MLPA value		FISH Result positive %[10]	
		T-Akt[1]	p-Akt[2]	Akt1	Akt2	AKT1	AKT2	AKT1	AKT2
Cancer tissue									
1	AC[3]	4	4	2	4	0.88	1.75	D	Amp[6] (53)
2	AC	2	2	0	2	1.01	1.39	D	poly-H[7] (81)
3	AC	2	2	2	1	1.28	1.16	poly-L (36)	poly-L[8] (17)
4	AC	2	2	2	1	1.13	0.96	poly-L (28)	D
5	AC	4	4	4	4	1.11	1.07	D[9]	D
6	AC	2	2	1	2	1.10	1.03	D	D
7	AC	1	0	1	0	1.09	1.06	D	D
8	AC	2	2	1	2	1.08	0.85	D	D
9	AC	1	1	0	1	1.07	0.97	D	D
10	AC	2	2	2	1	1.02	1.02	D	D
11	AC	1	1	1	0	1.02	1.02	D	D
12	AC	1	1	1	1	1.01	0.92	D	D
13	AC	1	1	1	1	1.00	0.96	D	D
14	AC	2	0	0	1	0.97	0.86	D	D
15	AC	1	1	1	0	0.93	1.03	D	D
16	AC	0	0	0	0	0.92	0.92	D	D
17	AC	0	0	0	0	0.82	0.84	D	D
18	SCC[4]	4	2	4	1	1.85	1.05	Amp (55)	D
19	SCC	4	4	0	2	0.88	1.26	D	poly-H (43)
20	SCC	2	2	0	2	1.03	1.26	D	poly-L (38)
21	SCC	2	0	0	2	1.09	0.96	D	D
22	SCC	2	2	0	0	1.08	1.09	D	D
23	SCC	4	1	1	4	1.06	1.06	D	D
24	SCC	1	0	1	0	1.02	0.96	D	D
25	SCC	1	1	0	1	0.96	0.89	D	D
26	SCC	0	0	0	0	0.94	0.88	D	D
27	SCC	2	1	2	2	0.90	0.91	D	D
28	SmCC[5]	4	4	4	0	1.47	1.05	poly-H (64)	D
29	SmCC	2	2	2	2	1.15	1.19	D	poly-L (22)
30	SmCC	1	0	1	1	0.91	0.84	D	D
Non-neoplastic tissue									
1	AC	0	0	0	0	0.97	0.96	D	D
2	AC	0	0	0	0	0.97	0.9	D	D
10	AC	1	0	0	1	0.97	0.96	D	D
16	AC	1	0	1	0	0.97	0.96	D	D
20	SCC	1	1	1	0	0.99	1.01	D	D
21	SCC	0	0	0	0	0.97	0.87	D	D
24	SCC	1	0	0	1	0.86	0.96	D	D
25	SCC	1	0	1	0	1.04	1.03	D	D
27	SCC	0	0	0	0	0.97	0.96	D	D
29	SmCC	1	0	0	1	1	0.97	D	D

Abbreviations: MLPA, multiplex ligation-dependent probe amplification; FISH, fluorescence in situ hybridization analysis; 1) T-Akt, total-Akt; 2) p-Akt, phosphorylated-Akt; 3) AC, adenocarcinoma; 4) SCC, Squamous cell carcinoma; 5) SmCC, Small cell carcinoma; 6) Amp, amplification; 7) poly-H, high level polysomy; 8) poly-L, low level polysomy; 9) D, disomy; 10) positive %, positive ratio of cells harboring gene increase to total cancer cells.

Table 4. Correlations between FISH and MLPA or immunohistochemistry

A

T-Akt[1] IHC

	+	-	total
***AKT1/2*-MLPA[4] (optimized cutoff)**			
+	10	0	10
-	44	6	50
total	54	6	60

sensitivity of IHC = 10/10 = 100%
specificity of IHC = 6/50 = 12%

B

p-Akt[2] IHC

	+	-	total
***AKT1/2*-MLPA (optimized cutoff)**			
+	10	0	10
-	34	16	50
total	44	16	60

sensitivity of IHC = 10/10 = 100%
specificity of IHC = 16/50 = 32%

C

Akt1 IHC[3]

	+	-	total
***AKT1*-MLPA[5] (optimized cutoff)**			
+	4	0	4
-	15	11	26
total	19	11	30

sensitivity of IHC = 4/4 = 100%
specificity of IHC = 11/26 = 42%

D

Akt2 IHC

	+	-	total
***AKT2*-MLPA[6] (optimized cutoff)**			
+	6	0	6
-	15	9	24
total	21	9	30

sensitivity of IHC = 6/6 = 100%
specificity of IHC = 9/24 = 38%

E

MLPA (standard cutoff)

	+	-	total
***AKT1/2*-FISH[7]**			
+	4	6	10
-	0	50	50
total	4	56	60

sensitivity of MLPA = 4/10 = 40%
specificity of MLPA = 50/50 = 100%

F

MLPA (optimized cutoff)

	+	-	total
***AKT1/2*-FISH**			
+	9	1	10
-	1	49	50
total	10	50	60

sensitivity of MLPA = 9/10 = 90%
specificity of MLPA = 49/50 = 98%

Abbreviations: 1) T-Akt, total-Akt; 2) p-Akt, phosphorylated-Akt; 3) IHC, positive/negative samples by immunohistochemistry; 4) AKT1/2-MLPA, positive/negative for "gain" in AKT1 and AKT2 genes by multiplex ligation-dependent probe amplification analysis; 5) AKT1-MLPA, positive/negative for "gain" in AKT1 gene by MLPA; 6) AKT2-MLPA, positive/negative for "gain" in AKT2 gene by MLPA; 7) AKT1/2-FISH, positive/negative for copy number increase in AKT1 and AKT2 genes by fluorescence in situ hybridization analysis. Each number indicates the number of the samples.

(43%). It is additionally possible that the quality of DNA extracted from FFPE versus fresh tumor tissue was lower, and/or that the custom-made probes had a lower efficiency compared to the commercially available probes. The overall sensitivity and specificity of the 'gain' by conventional MLPA versus CNIs detected by FISH were 40% (4/10) and 100% (50/50), respectively. These results indicate that measures need to be taken to enhance the sensitivity of the analysis, not only by trimming the section around the tumor cells, but also by optimizing the cut-off values for the particular analysis [7, 23]. In high-throughput screening for CNIs, sensitivity should take precedence over specificity, so a lower-cutoff value may be acceptable. We found that using the "average ± 2SE" to determine our revised cut-off value resulted in a fair correlation between MLPA and FISH. MLPA correctly found 9 of 10 samples with CNIs, with only one sample each of false-positive and false-negative. The sensitivity and specificity for the 'gain' classification were 90% (9/10) and 98% (49/50), respectively. Thus, by care-

fully calibrating the cutoff values, MLPA could serve as a screening method for the detection of CNIs that are present even in a limited fraction of the carcinoma cells in a tumor nodule. Although the setting of cutoff could vary depending on the sample group, i.e., the type of gene targeted and the number of samples, the sample group used in the current study appeared similar to those seen in other studies looking at lung carcinoma [16, 17]. Our samples consisted of 30 carcinoma specimens across three major histological types and included 10 samples of adjacent non-neoplastic tissues. Among these, amplification was found in one of the samples (3.3%) for each *AKT* gene, high-level polysomy for *AKT1* and *AKT2* in 3.3 and 6.7%, and low-level polysomy in 6.7% and 10% of the samples, respectively. As these are numbers seen in typical lung carcinoma samples, the revised MLPA cutoff values utilized here may apply more generally to the analysis of *AKT* CNIs. Although the MLPA analysis has been shown to be a robust technology for the analysis of various types of malignancies, the cutoffs employed in these analyses have remained unchanged [7, 22, 23, 27]. This is a crucial point, as in many invasive carcinoma samples, the tumor cell proportion should be low due to inevitable stromal contamination. In addition, tumor nodules often manifest intratumoral heterogeneity in the genetic aberrations occurring in individual cells [10]. In the present study, tissue samples had a broad range of CNI-positive carcinoma cell content, approximately 17 to 81%, which allowed us to evaluate the performance of MLPA in a heterogeneous collection of specimens. We found that this method was effective even when CNIs were present in only 17% of carcinoma cells (Case 3, *AKT2*). In our previous studies, we used commercially established probes and kits and found that MLPA could consistently identify gene amplification in samples containing as few as 30% positive cells, even using the conventional cutoff value [11]. In the FISH analysis, we often confine our analysis to smaller, more focused regions exhibiting higher protein expression. Similarly, the specificity of MLPA could be further increased by careful trimming and manual microdissection of the carcinoma tissues in the IHC-positive areas, which could contain a higher fraction of CNI-positive carcinoma cells. In the current study, we used larger tissue sections to screen for CNIs by MLPA, as well as to evaluate intratumoral heterogeneity by FISH. This would explain why one sample (case 4) exhibiting heterogeneity for CNI did not score as "gain" in MLPA despite exhibiting low-level polysomy by FISH.

Compared with SNP, array comparative genomic hybridization, and next-generation sequencing techniques, MLPA is relatively cheap, easy-to-perform, and allows the detection of CNIs in samples containing fragmented and smaller amounts of DNA, often encountered with FFPE tissues [10, 13, 28]. Since MLPA involves PCR amplification of DNA from many different cells, it has been considered mandatory to confirm these results with morphological methods such as FISH. However, the present study suggests that MLPA could be used solely as a high-throughput screening technique, provided the cutoff values are optimized to account for such variables as the potentially lower efficiency of custom-made probes and the low fraction of CNI-positive tumor cells in FFPE samples.

Although there are currently no Akt inhibitors approved for clinical use, there are a number in development in preclinical and clinical trials, including a pan-Akt inhibitor (MK-2206), an ATP-competitive Akt inhibitor (afuresertib) and a highly selective Akt1 inhibitor (A-674563), etc. [29, 30]. Given that there are few known oncogenes that are commonly amplified in lung cancers, including the *AKTs,* implementation of semi-comprehensive screening by MLPA using custom-made probes could facilitate the identification of the selected subset of patients who may benefit from a tailored therapy.

In conclusion, MLPA could be a feasible and effective ancillary method for large-scale screening of tumor samples and can accommodate both FFPE samples and the use of custom-made probes, provided that the criteria for classification is optimized. This method thus may be applicable for the screening of any relevant target oncogene and could more efficiently select candidate patients who may benefit from individualized therapies.

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Disclosure of conflict of interest

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