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Expression of Kita-Kyushu Lung Cancer Antigen-1 as Detected by a Novel Monoclonal Antibody in Gastric Cancer

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Abstract. *Background/Aim: Kita-Kyushu lung cancer antigen-1 (KK-LC-1) is a known cancer/testis antigen. Our group has previously shown KK-LC-1 gene expression in gastric cancer. However, could not be detected the KK-LC-1 protein due to the lack of an appropriate antibody. Here, we assessed our original monoclonal antibody (Kmab34B3) and, using it, assessed the expression of KK-LC-1 in gastric cancer. Patients and Methods: We evaluated an original monoclonal antibody against KK-LC-1 (Kmab34B3), and used this antibody to compare KK-LC-1 protein expression in tumour and non-tumour stomach cells from gastric cancer patients. Results: Kmab34B3 stained testicular germ cells, and tumour cells in nine out of 11 (82%) specimens. In non-tumorous areas, Kmab34B3 stained 13 out of 29 (45%) pyloric gland specimens. Furthermore, Kmab34B3 also stained intestinal metaplasia positive and negative areas. Conclusion: Kmab34B3 was able to detect KK-LC-1 protein within tumour cells and the pyloric gland where the gene has been shown to be expressed. Therefore, it might be an attractive tool for detecting KK-LC-1 expression in precancerous and cancerous stomach cells.*

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The Kita-Kyushu lung cancer antigen-1 (KK-LC-1), also known as CT83 and Cxorf61, is categorised as a cancer/testis antigen (CTA) which is expressed within various types of cancers at a particular rate, and is not expressed in normal tissues with the exception of germline tissues (1, 2). The corresponding gene is expressed in 82%, 54%, and 33% of gastric cancer (GC), breast cancer, and lung cancer, respectively (3-5).

KK-LC-1 has a 79% expression rate during the early stages of GC (6). Such finding indicates that KK-LC-1 expression occurs at the initiation of malignancy and is subsequently maintained. Furthermore, *KK-LC-1* has been shown to be expressed in premalignant lesions of the stomach (7), indicating again that KK-LC-1 expression occurs at premalignancy and is subsequently maintained up to malignancy and its progression, and that KK-LC-1 could be a predictive marker for GC occurrence. However, these studies evaluated the expression of the gene, but not the protein expression due to the lack of an appropriate antibody against KK-LC-1. Therefore, we generated a monoclonal antibody against KK-LC-1. In the present study, we evaluated whether this original monoclonal antibody accurately detected KK-LC-1 protein expression in tumour and non-tumour stomach cells.

Patients and Methods

Patients. Between March and September 2017, 27 patients underwent surgical resection for GC at the Department of Surgery, Kitasato University Medical Center in Kitamoto, Japan. We obtained both tumour as well as four non-tumour samples distal from the tumour area at random. All samples had sufficient mRNA quality based on *β-actin* gene expression levels.

Tissue specimens. Each specimen was immediately stored following collection at 4˚C overnight in RNAlater (Life Technologies, Carlsbad, CA, USA). Samples were subsequently stored at −80˚C until use. Tumor and non-tumor samples were subjected to hematoxylin-eosin staining in order to confirm the predominance of tumour cells within the area of the tumour, the lack of tumour cell contamination in non-tumorous areas and distinguish the sampled non-tumour areas into borderline, fundic or pyloric glands. Furthermore, we distinguished the area of pyloric glands into those containing intestinal metaplasia (IM) or not (non-IM).

KK-LC-1 gene expression. A QIACUBE and RNeasy Tissue Mini Kit (Qiagen, Hilden, Germany) was used to isolate total RNA from each sample according to the manufacturer's instructions. Total RNA was then converted to cDNA using oligo p(dN)6 random primers and Superscript III reverse transcriptase (Life Technologies). Assessment of RNA quality and *KK-LC-1* gene expression was performed as previously described (4). We obtained 11 tumour and four accompanying non-tumour specimens from GC patients, including six tumour specimens expressing the KK-LC-1 gene and five tumour specimens lacking *KK-LC-1* gene expression.

Immunohistochemical staining. The original anti-KK-LC-1 monoclonal antibody prepared in collaboration with CLEA Japan, Inc. (Tokyo, Japan), Kmab34B3, has been previously described (3). FFPE sections (3 μm) of adult human testis, gastric tumour, and normal gastric gland distal from the tumor mass were prepared. Antigen retrieval was carried out by autoclaving at 121˚C for 5 min in 10 mM citrate buffer solution at pH 6.0. Endogenous peroxidase was blocked using 6% H_2O_2. Kmab34B3 (1:80 for 1 h) and CL4762 (1:100 for 2 h; Abcam plc, Cambridge, UK) were used as primary antibodies for KK-LC-1 staining. Enzyme-labelled biotin-streptavidin techniques were applied using the Histofine Histostainer 48A automated immunohistochemistry slide staining system (Nichirei Bioscience, Chuo, Tokyo, Japan). Expression of KK-LC-1 was assessed by measuring staining intensity of tumour and stromal cells in GC tumors, and glands and stroma in gastric non-tumours.

Human rights statement and informed consent. The Human Ethics Review Committee of Kitasato University Medical Center, Japan (Approval No.: 29-18) approved the study protocol. All the experiments were performed in accordance with relevant guidelines and regulations. All patients signed an informed consent prior to resection of the tissue samples used in this study.

Statistical analysis. The Fisher's exact test was used for statistical analyses comparing KK-LC-1 expression and each factor. *p*-Values <0.05 were considered statistically significant. JMP14.0 (SAS institute Japan, Minato-ku, Japan) software was used for analyses.

Results

Original mAb evaluation. Our results indicated that the original mAb Kmab34B3 specifically stained germ cells at the lumen and basal lamina within seminiferous tubules. However, CL4762 stained only sperm heads of the luminal area and it remains unclear whether the antibody staining was intracellular or extracellular (Figure 1). Therefore, we used Kmab34B3 in this study for immunohistochemistry (IHC) of GC in order to evaluate KK-LC-1 expression.

KK-LC-1 expression within GC tumours. Figure 2 shows representative staining of KK-LC-1 at tumour sites using the

Table I. *The detection of Kita-Kyushu lung cancer antigen-1 and comparison of gene and protein expression in tumour cells*.

	Immunohistochemical staining		
	Positive	Negative	Rate (%)
Tumour area (n=11)	9	2	81.8
Gene expression			
Positive	6	0	100.0
Negative	3	2	60.0

Table II. *Detection of Kita-Kyushu lung cancer antigen-1 and comparison of gene and protein expression in non-tumour cells*.

	Immunohistochemical staining		
	Positive	Negative	Rate (%)
Non-tumour area (n=44)	16	28	36.4
Gene expression			
Positive	9	4	69.2
Negative	7	24	22.6
Pyloric gland (n=29)	13	16	44.8
Gene expression			
Positive	8	4	66.7
Negative	5	12	29.4
Border line (n=11)	3	8	27.3
Gene expression			
Positive	1	0	100.0
Negative	2	8	20.0
Fundic gland (n=4)	0	4	0.0
Gene expression			
Positive	0	0	–
Negative	0	4	0.0

Table III. *The detection of Kita-Kyushu lung cancer antigen-1 protein in pyloric gland with or without intestinal metaplasia*.

	Immunohistochemical staining			
	Positive	Negative	Rate (%)	*p*-Value
Intestinal metaplasia				
Positive	11	10	52.4	0.2378
Negative	2	6	25.0	

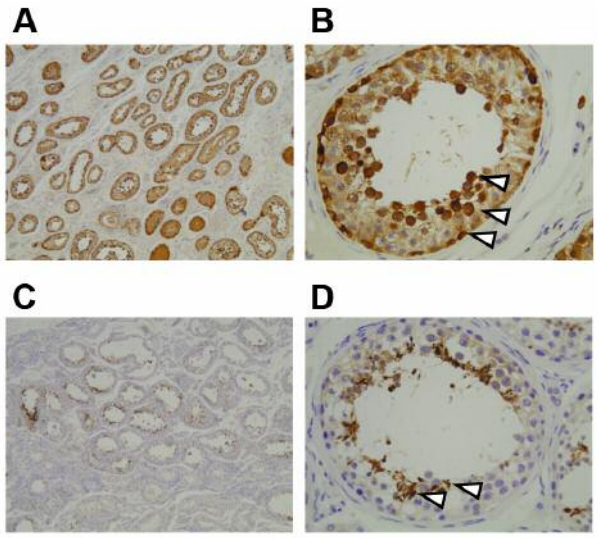

Figure 1. *Immunohistochemical staining for KK-LC-1 antigen in normal testis. Normal testis stained with Kmab34B3 (A and B) or CL4762 (C and D). Both antibodies stained seminiferous tubules internally and were negative for interstitial tissue (A and C). Kmab34B3 strongly stained spermatogenic cells on the basal lamina and spermatids (B). CL4762 stained only sperm heads (D). Arrow heads indicate cells strongly stained by antibodies. Magnification: 40× (A and C) and 400× (B and D).*

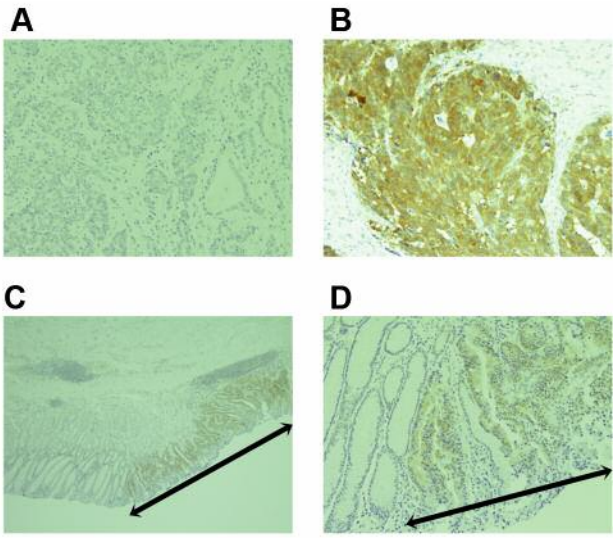

Figure 2. *Immunohistochemical staining of KK-LC-1 with Kmab34B3 of tumour cells in gastric cancer. Representative images of negative (A), strongly positive (B) and weakly positive (C and D) staining. Kmab34B3 stained all tumour cells but not stroma (B) and normal gastric glands adjacent to tumours (C and D). Double ended arrows indicate the staining area. Magnification: 200× (A, B and D) and 40× (C).*

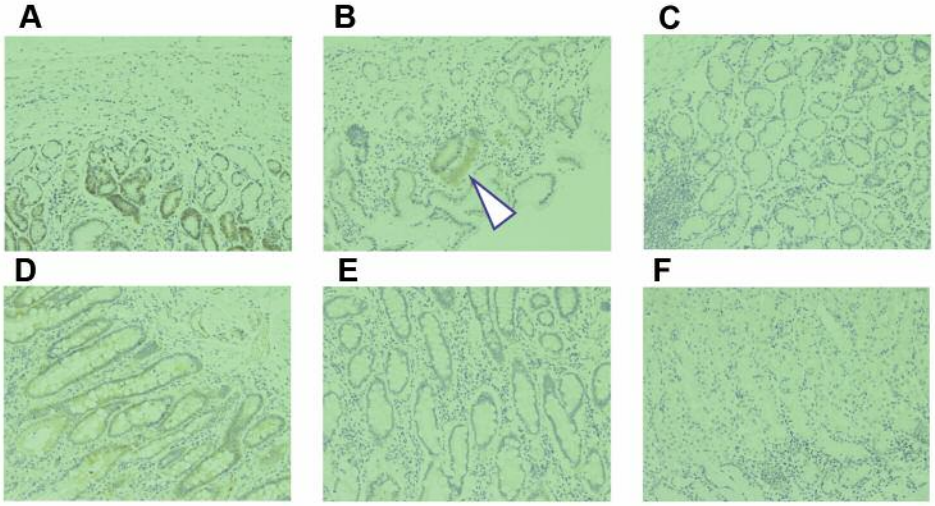

Figure 3. *Immunohistochemical staining of KK-LC-1 with Kmab34B3 in normal cells of gastric cancer stomach. Representative staining of pyloric glands (A-C), pyloric glands with intestinal metaplasia (D and E), and fundic glands (F). (A) Positive staining in pyloric glands; B) patchy positive staining (arrow head) in pyloric glands; (C) negative staining of pyloric glands; D) positive staining of all pyloric gland with intestinal metaplasia; (E) negative staining of pyloric glands with intestinal metaplasia; and (F) negative staining of fundic glands. Magnification: 200×.*

Kmab34B3 antibody. Kmab34B3 homogenously stained almost all tumour cells but not stromal cells (Figure 2A and B). Figure 2C and D show the front line of tumour cells. KK-LC-1 was detected only at tumour sites but not to adjacent non-tumour sites, including normal glands and stromal cells. Of the 11 GC tumours evaluated, KK-LC-1 protein was detected in nine specimens (81.8%), while *KK-LC-1* gene expression was detected in only six specimens (54.5%) (Table

I). A total of six tumour specimens were positive for both protein and gene expression, and two were negative for both. Alternatively, a total of three tumour specimens were positive for protein expression but negative for gene expression, whereas no tumour specimens were negative for the protein but positive for the gene expression (Table I).

KK-LC-1 expression in non-tumour areas. Figure 3 shows representative staining of KK-LC-1 with Kmab34B3 in non-tumour sites distal from tumours. KK-LC-1 staining was detected at pyloric gland cells (Figure 3A-C, Table II). We observed that KK-LC-1 was expressed in patches (Figure 3B). A total of eight pyloric gland specimens were positive for both protein and gene expression and 12 were negative for both. Alternatively, a total of five tumour specimens were positive for protein but negative for gene expression, whereas four were negative for protein but positive for gene expression (Table II). Furthermore, pyloric glands exhibiting intestinal metaplasia also expressed KK-LC-1 (Figure 3D and E, Table III). On the contrary, KK-LC-1 was completely undetected in fundic glands (Figure 3F, Table II).

Discussion

In this study, we demonstrated KK-LC-1 protein expression by using the novel anti-KK-LC-1 monoclonal antibody, Kmab34B3. KK-LC-1 has been shown to be strongly expressed in testicular germ cells, as KK-LC-1 is indeed a CTA (2, 8). Kmab34B3 strongly stained nearly all germ cells, from spermatogonia to spermatids. Monoclonal antibodies against CTAs have been reported to have similar staining as that shown here with Kmab34B3 (9-11). These results indicate that Kmab34B3 accurately detects KK-LC-1 in testicular germ cells. On the other hand, CL4762, another monoclonal antibody against KK-LC-1, did not stain testicular germ cells but strongly stained spermatids and sperm heads according to defined markers (12). Recent reports have shown that KK-LC-1 protein exists within the sperm nucleus (13). These results may indicate that these two monoclonal antibodies may recognise different isoforms of KK-LC-1 or conformationally different KK-LC-1 proteins.

Evaluation of KK-LC-1 expression with Kmab34B3 in tumour cells and normal cells from GC stomachs was performed and compared with gene expression data. In tumour cells, KK-LC-1 staining was observed in all specimens positive for *KK-LC-1* gene expression. Furthermore, we observed that the specimens, not expressing *KK-LC-1* gene, were stained with Kmab 34B3. These data indicate that KK-LC-1 detection by IHC using the Kmab34B3 antibody was more sensitive than the method used for detecting gene expression. In support, we have previously reported that the detection rate in breast cancer tumours increased by using IHC (3). Similarly, the KK-LC-1 expression rate in GC tumours may increase using IHC compared to gene expression analysis, as we have previously reported (4, 6, 7).

On the other hand, non-tumour cells of the pyloric gland did not show consistent results concerning KK-LC-1 expression between gene expression and protein staining. This inconsistency may have occurred because the expression of KK-LC-1 was heterogenous and patchy within the pyloric glands of the stomach. In fact, we observed single point KK-LC-1 staining as shown by an arrow head in Figure 3B. Therefore, the evaluation of KK-LC-1 expression via IHC may be more precise than that of its gene expression. Furthermore, we found no relation between KK-LC-1 expression and intestinal metaplasia using IHC. We have previously reported that with gene expression, KK-LC-1 was more frequently expressed in IM than in non-IM. It remained unclear whether the cells in which IM occurred expressed KK-LC-1 or not. Here, we observed by IHC that IM-cells partly expressed KK-LC-1, but no correlation between them was found (Table III). This indicates that KK-LC-1 is expressed at abnormal stages including atrophy, IM, and dysplasia but there is no correlation between them. Furthermore, Otsuka *et al*. have demonstrated that KK-LC-1 was rarely expressed in non-tumour cells of the stomach, and occurred only during atrophy in non-tumorous tissue (Otsuka *et al*., currently under submission). IM is thought to be a paracancerous rather than a precancerous indicator (14). Taking this into consideration, KK-LC-1 may be a novel stomach precancerous or paracancerous indicator for predicting whether gastric malignancy has occurred or will occur.

KK-LC-1 is considered to be a target for therapy and prediction of GC. Our results indicate that KK-LC-1 can be specifically detected with IHC using the Kmab34B3 antibody. IHC with Kmab34B3 antibody could be a companion diagnosis method for cancer therapy targeting KK-LC-1. Furthermore, Kmab34B3 could detect precancerous lesions of the stomach.

Conflicts of Interest

The Authors declare that they have no conflict of interest regarding this study.

Authors' Contributions

YT, TF, YI, TY, NH, and YK designed the study; TF, HN, and NK performed gene expression analyses; TF, NF, HO, and HY constituted and assessed original monoclonal antibodies; YT, TF, MC, and YN collected clinicopathological backgrounds and performed macroscopic evaluations; YT, TF, and HY performed microscopic evaluations; YT and TF performed statistical analyses; YT and TF wrote the main manuscript; All Authors reviewed the manuscript.

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References

1 Stevanovic S, Pasetto A, Helman SR, Gartner JJ, Prickett TD, Howie B, Robins HS, Robbins PF, Klebanoff CA, Rosenberg SA and Hinrichs CS: Landscape of immunogenic tumor antigens in successful immunotherapy of virally induced epithelial cancer. Science *356(6334)*: 200-205, 2017. PMID: 28408606. DOI: 10.1126/science.aak9510

2 Fukuyama T, Hanagiri T, Takenoyama M, Ichiki Y, Mizukami M, So T, Sugaya M, So T, Sugio K and Yasumoto K: Identification of a new cancer/germline gene, KK-LC-1, encoding an antigen recognized by autologous CTL induced on human lung adenocarcinoma. Cancer Res *66(9)*: 4922-4928, 2006. PMID: 16651449. DOI: 10.1158/0008-5472.CAN-05-3840

3 Kondo Y, Fukuyama T, Yamamura R, Futawatari N, Ichiki Y, Tanaka Y, Nishi Y, Takahashi Y, Yamazaki H, Kobayashi N and Watanabe M: Detection of KK-LC-1 protein, a cancer/testis antigen, in patients with breast cancer. Anticancer Res *38(10)*: 5923-5928, 2018. PMID: 30275220. DOI: 10.21873/anticanres.12937

4 Shida A, Futawatari N, Fukuyama T, Ichiki Y, Takahashi Y, Nishi Y, Kobayashi N, Yamazaki H and Watanabe M: Frequent high expression of Kita-Kyushu Lung Cancer Antigen-1 (KK-LC-1) in gastric cancer. Anticancer Res *35(6)*: 3575-3579, 2015. PMID: 26026129.

5 Shigematsu Y, Hanagiri T, Shiota H, Kuroda K, Baba T, Mizukami M, So T, Ichiki Y, Yasuda M, So T, Takenoyama M and Yasumoto K: Clinical significance of cancer/testis antigens expression in patients with non-small cell lung cancer. Lung Cancer *68(1)*: 105-110, 2010. PMID: 19545928. DOI: 10.1016/j.lungcan.2009.05.010

6 Futawatari N, Fukuyama T, Yamamura R, Shida A, Takahashi Y, Nishi Y, Ichiki Y, Kobayashi N, Yamazaki H and Watanabe M: Early gastric cancer frequently has high expression of KK-LC-1, a cancer-testis antigen. World J Gastroenterol *23(46)*: 8200-8206, 2017. PMID: 29290656. DOI: 10.3748/wjg.v23.i46.8200

7 Fukuyama T, Futawatari N, Yamamura R, Yamazaki T, Ichiki Y, Ema A, Ushiku H, Nishi Y, Takahashi Y, Otsuka T, Yamazaki H, Koizumi W, Yasumoto K and Kobayashi N: Expression of KK-LC-1, a cancer/testis antigen, at non-tumour sites of the stomach carrying a tumour. Sci Rep *8(1)*: 6131, 2018. PMID: 29666402. DOI: 10.1038/s41598-018-24514-9

8 Futawatari N, Fukuyama T, Yamamura R and Kobayashi N: Helicobacter pylori infection induces gastric cancer and the cancer/testis antigens expression. J Infect Dis Ther *5(6)*: e1000345, 2017. DOI: 10.4172/2332-0877.1000345

9 Daudi S, Eng KH, Mhawech-Fauceglia P, Morrison C, Miliotto A, Beck A, Matsuzaki J, Tsuji T, Groman A, Gnjatic S, Spagnoli G, Lele S and Odunsi K: Expression and immune responses to MAGE antigens predict survival in epithelial ovarian cancer. PLoS One *9(8)*: e104099, 2014. PMID: 25101620. DOI: 10.1371/journal.pone.0104099

10 Odunsi K, Jungbluth AA, Stockert E, Qian F, Gnjatic S, Tammela J, Intengan M, Beck A, Keitz B, Santiago D, Williamson B, Scanlan MJ, Ritter G, Chen YT, Driscoll D, Sood A, Lele S and Old LJ: NY-ESO-1 and LAGE-1 cancer-testis antigens are potential targets for immunotherapy in epithelial ovarian cancer. Cancer Res *63(18)*: 6076-6083, 2003. PMID: 14522938.

11 Simpson AJG, Caballero OL, Jungbluth A, Chen YT and Old LJ: Cancer/testis antigens, gametogenesis and cancer. Nat Rev Cancer *5(8)*: 615-625, 2005. PMID: 16651449. DOI: 10.1158/0008-5472.CAN-05-3840

12 Djureinovic D, Fagerberg L, Hallström B, Danielsson A, Lindskog C, Uhlén M and Pontén F: The human testis-specific proteome defined by transcriptomics and antibody-based profiling. Mol Hum Reprod *20(8)*: 476-488, 2014. PMID: 24598113. DOI: 10.1038/nrc1669

13 de Mateo S, Castillo J, Estanyol JM, Ballesca JL and Oliva R: Proteomic characterization of the human sperm nucleus. Proteomics *11(8)*: 2714-2726, 2011. PMID: 21630459. DOI: 10.1002/pmic.201000799

14 Yao T, Ueyama H and Hirahashi M: High risk group of the gastric carcinogenesis from the pathological point of view: with special reference to mucin phenotypic expression of the carcinoma and its background mucosa. Stomach Intestine *44(9)*: 1423-1431, 2009. DOI: 10.11477/mf.1403101740

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