Original article

Prevalence and type of *KRAS* mutations in endometrial endometrioid carcinoma

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Background: *KRAS* encodes a small G-protein that involves cell proliferation, differentiation, and survival.
Objectives: To determine the prevalence and type of *KRAS* mutations in Southern Thai patients with endometrial endometrioid carcinoma as well as their correlation with clinicopathological variables.
Methods: A total of 190 patients with endometrioid carcinoma were analyzed for *KRAS* exon 2 mutations using direct sequencing. The statistical correlation of *KRAS* mutations with clinicopathogical variables was also evaluated using the Chi-square or Fisher exact test.
Results: *KRAS* mutations were detected in 17.4% (33/190) of cases. All of them were missense mutations and 72.7% (24/33) occurred in hotspot codons 12 and 13. Of these mutations, 30 tumors presented as single mutations including; 16 mutations in codon 12 (p.G12C, p.G12D, p.G12S, and p.G12V), 5 mutations in codon 13 (p.G13C, and p.G13D), and 9 rare mutations in the flanking regions of the hotspot (p.E3K, p.E3G, p.V14I, p.G15S, p.H27Y, p.D30N, p.D33G, and p.P34S). Additionally, 3 tumors presented as double mutations in codon 12 as well as in codons 2, 6, and 7 (p.G12D and p.T2I, p.G12C and p.F6L, and p.G12D and p.E7M). *KRAS* mutations were often found in patients with histologic grades 1 and 2 and FIGO stages I. There was a significant relationships between the presence of *KRAS* mutations and FIGO staging (P = 0.041), but no any mutations were significant correlated with other clinicopathological variables such as body mass index, myometrial invasion, LVSI, and synchronous ovarian cancer/ovarian metastasis.
Conclusion: This study suggests that the presence of *KRAS* mutations as single or double mutations would be a relatively common early event in endometrial carcinogenesis among this subgroup of Thai patients.

Keywords: Endometrioid carcinoma, *KRAS*, mutation, double mutation.

Endometrial carcinoma is the third most common gynecologic malignancy in the Thai population after cervical cancer and ovarian cancer, with an age standardized incidence rate (ASR) of 4.5 per 100,000 women-years.[1] Most patients are menopausal, and their average age at diagnosis is approximately 60 years. Endometrial cancer is generally classified into two distinctive pathogenetic subtypes that differ in histological and molecular characteristics.[2-4] Type-I tumors are associated with unopposed estrogenic stimulation. They are endometrioid carcinomas and are frequently preceded by endometrial hyperplasia. Moreover, they are the most common type, comprising approximately 80.0% of endometrial carcinomas, and tend to be low- or intermediate-grade tumors.[5] Type-II tumors, on the other hand, result from a sequence of genetic alterations occurring in atrophic endometrium. They are more likely to be high-grade lesions of serous or clear-cell histologic type.[3,6] The

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Received: September 18, 2020
Revised: October 27, 2020
Accepted: December 30, 2020

prognosis of type-II tumors is generally poor, with a high risk of both relapse and metastasis.

Endometrioid carcinoma is the most common type of endometrial carcinomas. The Cancer Genome Atlas (TCGA) has studied the molecular alterations of endometrioid carcinomas and detected frequent mutations in *PTEN*, *CTNNB1*, *PIK3CA*, *ARID1A*, and *KRAS* as well as novel mutations in the *SWI/SNF* gene *ARID5B*.[7] *KRAS* is a proto-oncogene, and it encodes a 21 kDa guanine nucleotide-binding protein (G-protein) that functions as a molecular switch in the intracellular transduction pathway involving cell proliferation and differentiation.[8, 9] The oncogenic mutations of the *KRAS* gene result in constitutive signal transduction pathways, which subsequently affect upregulated cell proliferation, survival, and cancer progression. *KRAS* mutations have been identified in around 10.0 – 30.0% of endometrioid carcinomas.[10 - 14] They are predominantly found in codons 12 and 13 of exon 2 and rarely in codon 61. Mutations of the *KRAS* have been suggested as a molecular assessment of the depth of myometrial invasion in endometrial cancer.[15] *KRAS* mutations also are detected in endometrial hyperplasia, which suggests that *KRAS* mutations may be a relatively early event in endometrial carcinogenesis.[10, 16] Furthermore, recent studies have shown that the combination therapy of mitogen-activated extracellular kinase (MEK) inhibitors plus anti-estrogen agents may be necessary to improve response rates in patients with *KRAS* mutant endometrial cancer.[17] Thus, the study of molecular factors involved in its carcinogenesis would be helpful in achieving both prognosis stratification as well as optimized treatment options. The present study aimed to determine the prevalence and type of *KRAS* mutations in Southern Thai patients with endometrial endometrioid carcinoma as well as their correlation with clinicopathological variables.

Materials and methods

Patients

A retrospective cohort of 190 women, who underwent hysterectomy for endometrial cancer at Songklanagarind Hospital between 2008 and 2012, was assembled. This study has been approved by the Ethics Committees of Prince of Songkla University (EC 56-190-05-1-3). All patients were diagnosed as endometrioid carcinoma of uterus. Inclusion criteria of selected samples, all patients are Thai. Clinical and pathological data are completely recorded. Tumor tissues are enough quantity to extract DNA in the concentration of 50 - 500 ng/μl.

Clinical and pathological data for cohort, including age, body mass index (BMI), parity, history of diabetes mellitus, hypertension, tumor grade, FIGO stage, myometrial invasion, lymphovascular invasion (LVSI), uterine cervical involvement, lymph node involvement, and synchronous ovarian cancer/ovarian metastasis were retrieved from the electronic medical records system.The sample size was calculated using the following formula:

$$n = \frac{Z^2 P (1 - P)}{d^2}$$

Where n = sample size, Z = Z statistic for the level of confidence, P = expected prevalence, and d = error of estimation.[18, 19] For conventional 95% confidence level, the z value is 1.96. *KRAS* mutations have been identified around 10.0 – 30.0% of endometrioid carcinomas. The calculated sample size was done using $P = 0.15$, $Z = 1.96$, and $d = 0.05$. The calculated sample size estimation was 195 patients. There were 5 samples with poor quality of DNA. Thus, overall sample size for this study was data from 190 patients.

DNA extraction and PCR analysis

A total of 190 formalin-fixed, paraffin-embedded tumor samples were selected after reviewing the hematoxylin and eosin (H & E)-stained slides. The tumor areas were microdissected on a corresponding unstained slide and transferred to an Eppendorf tube for DNA isolation. The DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The polymerase chain reaction (PCR) for KRAS in exon 2 was performed using a two-primer set. The primers for an amplicon size of 290 bp were F1 5'- GTACTGGTGGAGTATTTGAT-3' and R1 5'-ACTCATGAAAATGGTCAGAG-3'. [20] The primers for an amplicon size of 220 bp were F2 5'-CTTATGTGTGACATGTTCT-3' and R2 5'-AGAATGGTCCTGCACCAGTA-3'. [21] All samples were screened for the first PCR amplification with the primer-set consisting of F1 and R1. The amplified samples that were negative were re-amplified using the primer-set involving F2 and R2. The quality of DNA was amplified with the primer of the beta globin gene. The primers for the PCR products with a size of 268 bp were PC04 5'-CAACTTCATCCACGTT

CACC-3' and GH20 5'-GAAGAGCCAAGGACA GGTAC-3'. [22] The PCR conditions were as follows: 94°C for 4 min, then 40 cycles at 94°C for 45 sec, at 51°C (KRAS) and 55°C (beta globin) for 45 sec, and at 72°C for 1 min, with a final extension at 72°C for 7 min. The PCR products underwent agarose gel electrophoresis and were stained with ethidium bromide for size verification.

Sequencing analysis

The purified PCR products from the genomic DNA were applied for sequencing reaction in both directions using the ABI Prism 3500 Genetic Analyzer (Applied Biosystems). The sequencing data were analyzed using the sequencing analysis software 6.

Statistical analysis

The statistical analysis was performed using the R program. The relationship between clinicopathological variables and the presence of *KRAS* mutations was determined using the Chi-square or Fisher exact test. Statistical significance was set at a *P* - value of < 0.05.

Results

The median age of the 190 patients with endometrioid carcinoma was 57 years, ranging from 26 to 86 years, and the majority being > 40 to ≤ 60 years of age (n = 110, 57.9%). Their body mass index (BMI) ranged from 17 to 51 kg/m^2 (median BMI = 27.4 kg/m^2); 76 patients (40.0%) were overweight (BMI ≥ 25 - 30 kg/m^2), and 46 patients (24.2%) met the criteria for obesity (BMI > 30 kg/m^2). Fifty-two patients (27.4%) had diabetes mellitus, and 87 patients (45.8%) suffered from hypertension. Additionally, 157 patients (82.6%) were graded as G1 and G2, and 33 patients (17.4%) were graded as G3. The distribution by FIGO stage was: stage I in 139 patients (73.1%), stage II in 18 patients (9.5%), and stages III and IV in 33 patients (17.4%). The majority of the patients (n = 178, 93.7%) had undergone lymph node dissection or sampling, and 21 patients (11.1%) showed lymph node involvement. Synchronous ovarian cancer or ovarian metastasis was found in 10 patients (5.3%). The clinicopathological variables are summarized in Table 1.

Table 1. Clinical and pathological characteristics of 190 patients with endometrioid carcinoma.

Characteristics	Number of patients (%)
Age (years)	
≤ 40	16 (8.4)
> 40 - ≤ 60	110 (57.9)
> 60	64 (33.7)
BMI (kg/m^2)	
< 25	68 (35.8)
≥ 25 - 30	76 (40.0)
> 30	46 (24.2)
Parity	
Nulliparous	60 (31.6)
Multiparous	130 (68.4)
DM	
No	138 (72.6)
Yes	52 (27.4)
Hypertension	
No	103 (54.2)
Yes	87 (45.8)
Histologic grade	
G1	98 (51.6)
G2	59 (31.0)
G3	33 (17.4)
FIGO Stage	
I	139 (73.1)
II	18 (9.5)
III	30 (15.8)
IV	3 (1.6)

Table 1. (Con) Clinical and pathological characteristics of 190 patients with endometrioid carcinoma.

Characteristics	Number of patients (%)
Myometrial invasion	
No	25 (13.1)
< 50.0%	94 (49.5)
≥ 50.0%	71 (37.4)
LVSI	
No	140 (73.7)
Yes	50 (26.3)
Uterine cervical involvement	
No	175 (92.1)
Yes	15 (7.9)
Lymph node involvement	
No	157 (82.6)
Yes	21 (11.1)
None	12 (6.3)
Synchronous ovarian cancer/ovarian metastasis	
No	180 (94.7)
Yes	10 (5.3)

BMI = body mass index, DM = diabetes mellitus, LVSI = lymph vascular space invasion

Frequencies of KRAS mutations and its association with the clinicopathological variables of endometrioid carcinoma

Mutations in *KRAS* were detected in 17.4% (33/190) of cases. All were amino acid substitutions (missense mutation), and 72.7% (24/33) of mutations occurred in hotspot codons 12 and 13. Of these mutations, 21 tumors presented as single mutations in codon 12 or 13, and 3 tumors presented as double mutations in codon 12 as well as codons 2, 6, or 7. The most frequent mutations of variants in codons 12 and 13 were p.G12D (c.35G>A) and p.G13D (c.38G>A), respectively. Approximately a quarter of cases (27.3%, 9/33) presented with single mutations at non-hotspot sites of exon 2 such as 3, 14, 15, 27, 30, 33, and 34 (Figure 1). The frequency of *KRAS* missense mutations identified in endometrioid carcinomas is shown in Table 2.

Table 2. Pattern of *KRAS* missense mutations detected in 33 patients with endometrioid carcinoma.

KRAS mutation	Nucleotide change	Protein change	Mutation frequency (%)
Codon 12	c.34G>A	p.G12S	1 (3.0)
	c.34G>T	p.G12C	3 (9.1)
	c.35G>A	p.G12D	9 (27.3)
	c.35G>T	p.G12V	3 (9.1)
Codon 13	c.37G>T	p.G13C	1 (3.0)
	c.38G>A	p.G13D	4 (12.1)
Codon 12, 2	c.35G>A	p.G12D	1 (3.0)
	c.5C>T	p.T2I	
Codon 12, 6	c.34G>T	p.G12C	1 (3.0)
	c.16T>C	p.F6L	
Codon 12, 7	c.35G>A	p.G12D	1 (3.0)
	c.19G>A	p.E7M	
Codon 3	c.7G>A	p.E3K	1 (3.0)
	c.8A>G	p.E3G	1 (3.0)
Codon 14	c.40G>A	p.V14I	1 (3.0)
Codon 15	c.43G>A	p.G15S	1 (3.0)
Codon 27	c.79C>T	p.H27Y	1 (3.0)
Codon 30	c.88G>A	p.D30N	1 (3.0)
Codon 33	c.98A>G	p.D33G	2 (6.1)
Codon 34	c.100C>T	p.P34S	1 (3.0)

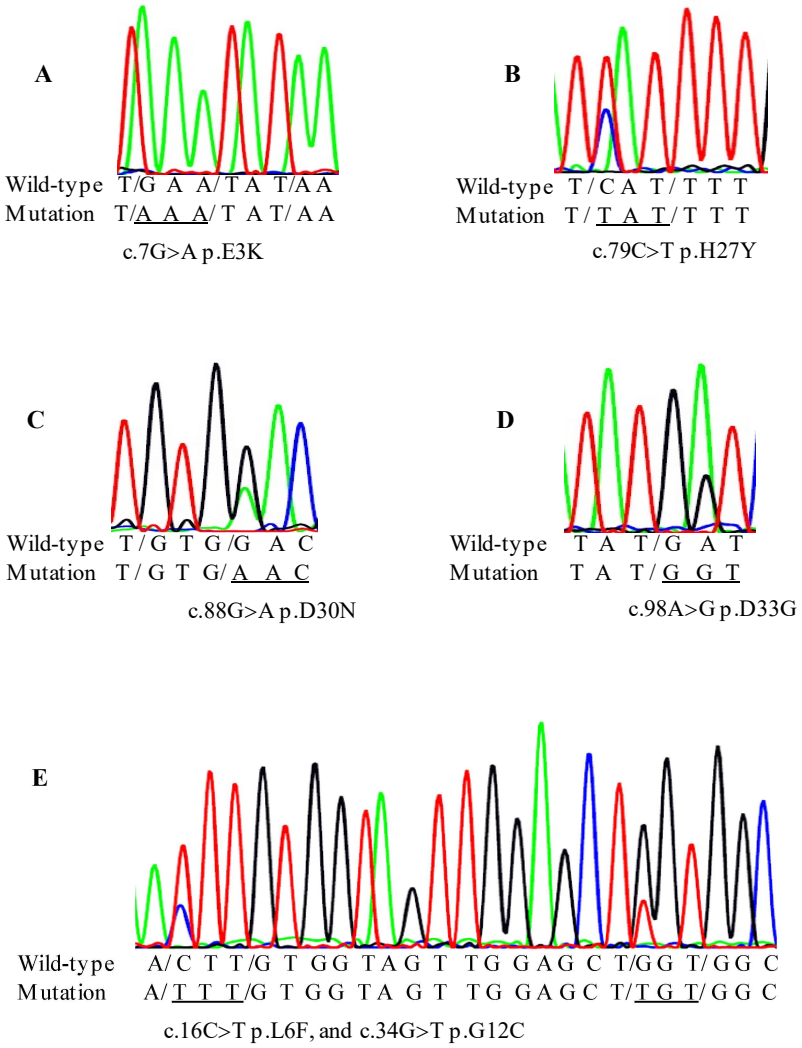

Figure 1. Sequencing chromatograms showing *KRAS* mutations in exon 2 of endometrioid carcinoma specimens. Point mutations were observed at codons 3, p.E3K **(A)**; 27, p.H27Y **(B)**; 30, p.D30N **(C)**; and 33, p.D33G **(D)**, and double mutations at codons 6 and 12 (p.L6F and p.G12C) **(E)**.

It was observed that 69.7% (23/33) of patients with *KRAS* mutations were ≤ 60 years and the remaining 30.3% (10/33) > 60 years of age; however, no statistically significant correlation was detected. The presence of *KRAS* mutations showed a significant association with FIGO staging ($P = 0.041$), but no any mutations were significant correlation with other clinicopathological variables like BMI, parity, histologic grade, depth of myometrial invasion, LVSI, uterine cervical and lymph node involvement, and synchronous ovarian cancer/ovarian metastasis (Table 3). With respect to prognosis, 3 out of 33 patients with *KRAS* mutations died of cancer and one patient had progression of disease. Additionally, there were 10 patients had personal or familial history of thyroid disease or carcinomas of the pancreas, colon, ovary, uterus, and cervix (Table 4).

Table 3. Correlation between clinicopathological features and *KRAS* mutation status in 190 patients with endometrioid carcinoma.

Clinicopathological features	Total	*KRAS* mutation positive (%)	*KRAS* mutation negative (%)	*P* - value
All patients	190	33 (17.4)	157 (82.6)	
Age (yrs)				
Range	26 - 86	27 - 86	26 - 79	
Average age	56.1	56.4	56.1	0.651
≤ 60	126	23 (18.3)	103 (81.7)	
> 60	64	10 (15.6)	54 (84.4)	
BMI (kg/m²)				0.411
< 25	68	14 (20.6)	54 (79.4)	
≥ 25 - 30	78	13 (16.7)	65 (83.3)	
> 30	44	6 (13.6)	38 (86.4)	
Parity				0.515
Nulliparous	60	11 (18.3)	49 (81.7)	
Multiparous	130	22 (16.9)	108 (83.1)	
Diabetes mellitus				0.383
No	138	26 (18.8)	112 (81.2)	
Yes	52	7 (13.5)	45 (86.5)	
Hypertension				0.966
No	103	18 (17.5)	85 (82.5)	
Yes	87	15 (17.2)	72 (82.8)	
Histologic grade				0.167
G1	98	19 (19.4)	79 (80.6)	
G2	59	12 (20.3)	47 (79.7)	
G3	33	2 (6.1)	31 (93.9)	
FIGO stage				0.041*
I	139	30 (21.6)	109 (78.4)	
II	18	1 (5.6)	17 (94.4)	
III and IV	33	2 (6.1)	31 (93.9)	
Myometrial invasion				0.959
No	25	4 (16.0)	21 (84.0)	
< 50%	94	16 (17.0)	78 (83.0)	
≥ 50%	71	13 (18.3)	58 (81.7)	
LVSI				0.891
No	140	24 (17.1)	116 (82.9)	
Yes	50	9 (18.0)	41 (82.0)	
Uterine cervical involvement				1.000
No	175	31 (17.7)	144 (82.3)	
Yes	15	2 (13.3)	13 (86.7)	
Lymph node involvement				0.503
No	157	30(19.1)	127 (80.9)	
Yes	21	2 (9.5)	19 (90.5)	
None	12	1 (8.3)	11 (91.7)	
Synchronous ovarian cancer/ovarian metastasis				0.686
No	180	31 (17.2)	149 (82.8)	
Yes	10	2 (20.0)	8 (80.0)	

*$P < 0.05$

Table 4. *KRAS* mutation and clinical data of endometrioid carcinoma.

Patient number	Nucleotide change	Amino acid change	Personal history	Familial history	Histologic grade	FIGO stage	PFS
1	c.7G>A	p.E3K			1	IB	PD
2	c.35G>A	p.G12D			1	IA	PD
3	c.5C>T	p.T2I			1	IB	PD
4	c.35G>A	p.G12D			1	IB	PA
5	c.88G>A	p.D30N	Colon cancer, Pancreas cancer		2	IA	A
6	c.34G>T	p.G12C	Ovarian cancer		1	IA	A
7	c.35G>A	p.G12D	Ovarian cancer		2	IB	A
8	c.100C>T	p.P34S		Sister: uterus cancer	2	IB	A
9	c.38G>A	p.G13D		Mother: colon cancer, Uterine cervical cancer	2	IA	A
10	c.37G>T	p.G13C		Grandmother and sister: colon cancer	1	1B	A
11	c.35G>A	p.G12D		Sister: uterine cervical cancer	2	IA	A
12	c.43G>A	p.G15S		Aunt: uterus cancer	1	IB	A
13	c.98A>G	p.D33G	Grave's disease		1	IB	A
14	c.98A>G	p.D33G	Thyroid tumor		1	IB	A
15	c.8A>G	p.E3G	Fatty liver		2	IB	A
16	c.35G>A	p.G12D	Ovary: thecoma		1	IB	A
17	c.35G>A	p.G12D			1	IB	A
18	c.35G>A	p.G12D			3	IIIC2	A*
19	c.35G>A	p.G12D			1	II	A
20	c.35G>A	p.G12D			1	IB	A
21	c.34G>A	p.G12S			3	IA	A
22	c.34G>T	p.G12C			1	IA	A
23	c.34G>T	p.G12C			1	IB	A
24	c.35G>T	p.G12V			2	IB	A
25	c.35G>T	p.G12V			1	IA	A
26	c.35G>T	p.G12V			1	IB	A
27	c.38G>A	p.G13D			1	IA	A
28	c.38G>A	p.G13D			2	IA	A
29	c.38G>A	p.G13D			2	IIIC	A
30	c.34G>T c.16T>C	p.G12C p.F6L			1	IA	A
31	c.35G>A c.19G>A	p.G12D p.E7M			2	IA	A
32	c.40G>A	p.V14I			2	IA	A
33	c.79C>T	p.H27Y			2	IA	A

PFS: progression free survival 5-year, PD: progression/died of disease, PA: progression of disease, alive at 5-year, A: alive without disease, FIGO: International Federation of Gynecology 2009. A* A patient had follow-up data for 3 years.

Discussion

Mutations of the *KRAS* gene have been detected in several types of tumors such as carcinomas of pancreas, colon, lung, breast, and endometrium. This study detected a 17.4% (33/190) frequency of mutations in exon 2 of *KRAS* in endometrioid adenocarcinoma. This finding is in line with those of previous studies, which have reported frequencies ranging from 10.0 - 30.0%.[3, 10, 23] It was also observed that the most common location of point mutations occurred in *KRAS* codon 12 rather than codon 13.[24 - 25] Moreover, the present study showed that the *KRAS* mutations in exon 2 were most frequently located at codons 12 (19/33) and 13 (5/33), and less frequently at codons 3, 14, 15, 27, 30, 33, and 34 (9/33). *KRAS* codons 12 and 13, coding for two adjacent glycines are the most common sites of oncogenic activation.[26] Any mutation resulting in amino acid alterations at these codons, which encode amino acids adjacent to the GDP/GTP binding pocket, reduces or abolishes GTPase activating proteins (GAPs)-mediated GTP hydrolysis and locks the protein in an active state, namely the GTP-bound state.[27] The GTP-bound activated KRAS interacts with various effector pathways resulting in a much higher proliferation of cancer cells. In accordance with our data, previous studies have typically identified an incorporation of other amino acids, most commonly aspartic acid and valine at codon 12 (p.G12D and p.G12V) and aspartic acid at codon 13 (p.G13D).[28 - 31] These amino acid alterations cause the projection of larger amino acid side chains into the GDP/GTP binding pocket of the protein that interfere with the geometry of the transition state, during which the GTP hydrolysis is catalyzed.[32]

Interestingly, among the 19 missense mutations of the *KRAS* gene located at codon12 in this study, the presence of double mutations was detected in 3 samples–codons 12 and 2 (p.G12D and p.T2I), codons 12 and 6 (p.G12C and p.F6L), and codons 12 and 7 (p.G12D and p.E7M). A previous study reported identifying *KRAS* mutations in codons 12 and 22 (p.G12S and p.Q22R) occurring in the same allele of a colon cancer case.[33] A double mutation at codons 19 and 20 (p.L19F and p.T20A) in a colorectal cancer case has also been reported and the in vitro synergistic effects on transformation of this co-mutation of the *KRAS* have been described.[34]

The present study revealed *KRAS* point mutation sites outside of codons 12 and 13; they were located at codons 3, 14, 15, 27, 30, 33, and 34. Previous studies have reported mutation sites not located solely in hotspot codons 12 and 13 but also in codons 15, 18, 20, 27, 30, and 31 in colorectal cancerous tissues and adrenal tumors.[35 - 37] It is known that the *KRAS* mutation at codon 15 decreases the binding ability of the mutant KRAS protein to GTPase activation proteins in colorectal cancers.[36] However, the exact mechanism of any *KRAS* mutation outside hotspot codons remains largely unknown.

Point mutations in the *KRAS* gene are also found in endometrial hyperplasia, suggesting they are early events in endometrial carcinogenesis.[10, 16] Furthermore, *KRAS* mutations present in endometrial hyperplasia next to carcinoma as well as in adjacent carcinoma.[14] Additionally, *KRAS* mutations are reported to play a role in the formation of superficial epithelial changes in the corresponding endometrioid carcinomas.[38] These findings lend support to the idea that *KRAS* status can be a prognostic marker in the transition from pre-malignant to malignant cell status. The prognostic impact of the association between *KRAS* mutations and clinicopathological features in endometrial cancers remains inconclusive. It has been shown that the presence of *KRAS* mutations in endometrioid carcinoma is significantly associated with lymph node metastases and poor survival among patients above 60 years of age.[24] Yet, only a borderline significant correlation between the presence of submicroscopic myometrial invasion and depth of myometrial invasion has been observed in FIGO stage I endometrial cancer patients.[15] Even though *KRAS* mutations are significantly associated with endometrioid subtype, a low tumor grade, and obesity, they have not been found to correlate with metastatic lesions and clinical outcome.[39] The present study's findings show that *KRAS* mutations are significantly correlated with FIGO early stage. Trend of *KRAS* mutations occur mostly in patients with histologic grades 1 and 2. These findings support the role of *KRAS* mutations in early involvement in endometrial carcinogenesis. However, some previous studies did not find correlation of *KRAS* mutations with any clinicopathological variable.[12, 25, 40] Given this observation, the study supports data that the patients with *KRAS* mutations seem to be associated with thyroid disease or cancers of colon, pancreas, cervix, and ovary. The frequently mutated *KRAS* gene in these cancers has been previously described.[41]

Conclusion

This study provides preliminary evidence that the presence of single or double *KRAS* mutations would be a relatively common early event in endometrial carcinogenesis in this subgroup of Thai patients.

Acknowledgements

We would like to thank Ms. Paradee Prechawittayakul from the Cancer Information Center for her help in searching and collecting the patient data. We greatly appreciate to Dr. Sariya Dechamethakun from Molecular Laboratory as well as the staff from the Epidemiology Unit, Faculty of Medicine, Prince of Songkla University for their assistance with the statistical analysis. The authors thank the staff of the International Affairs Office, Faculty of Medicine, Prince of Songkla University for the editing English manuscript. This study was supported by the Prince of Songkla University Fund.

Conflicts of interest

The authors declare no conflict of interest.

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